Immunotoxicological effects of mouse CpG ODN in lupus-prone NZB/NZW F1 mice

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Immunotoxicological effects of mouse CpG ODN in lupus-prone NZB/NZW F1 mice

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Health, as officially defined by the World Health Organization, means “A state of complete physical, mental, and social well-being, not merely the absence of disease or infirmity.” Daringly, I would like to say it “Having a power of defending themselves from attacks from the outside.” Where does the power come from? Our body is sensitive to changes in the external environment, and always maintains homeostasis. Interestingly, like a movie “Sleeping with the enemy,” Normal flora lives in harmony within our body to the nearest. Through a peaceful coexistence, we obtain basic immunity. Immunity is a state of having sufficient biological defenses to avoid infection, disease, or other unwanted biological invasions. Perhaps the current healthiness shows the potential to protect us from the external environment by healthy immune system?

The transition from “Nursing” to “Basic medicine” was an important turning point of my life. To encounter immunity, a difficult and a strange word, I began Basic Medical Microbiology in the spring of 2005. “Microbiology and Immunology” like “Alice in Wonderland” were inextricable mazes to me. As I was in the Wonderland of Microbiology and Immunology, one person was a navigator for me. I would like to express my sincere gratitude to my advisor, Dr. Soo-Ki Kim who has kindly led me to the way that I learned my major field of study and practiced animal experiments about it. I would like to thank the doctoral dissertation committee, Dr. Soon Hee Jung, Dr. Choon Hee Chung, Dr. Kum Whang, and Dr. Tae-Young Kang, who gave me endless affection and attention.

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Celebrating the last night, December 31, 2008

Written by Bo Hwan Kim

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ABSTRACT

Immunotoxicological effects of mouse CpG ODN in lupus-prone NZB/NZW F1 mice

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Background and purpose: CpG ODNs as vaccine adjuvant or immunotherapeutic are widely exploited in preclinical or clinical therapy for infection, allergy and cancer. Despite the versatile applicability of CpG ODN, the safety and toxicity of CpG ODNs were not well established. Toward this, I investigated whether optimal or escalated dosing of CpG ODN treatment would affect immunotoxicological parameters reflecting autoimmunity status in NZB/NZW F1 mice. **Methods and materials:** To identify immunotoxicological effects of CpG ODN, two consequtive experiments in lupus-prone mice were perfomed: 1) comparisons among saline only, non-CpG and mouse CpG ODN (10 μg)-treated groups for 4 weeks and 2) comparisons among escalated dose (0, 10, 30, 50 μg) of mouse CpG ODN-treated groups for 8 weeks. Autoimmune parameters to gauge the immunotoxicity of CpG ODNs were designed as follows: 1) nonspecific immunotoxicological profiles: BW, organ/BW ratio, and routine hematology and/or serology, 2) specific autoimmune organ profiles: lupus nephritis and proteinuria level, 3) autoimmune serology marker / autoantibody marker: titer of auto-dsDNA Ab and auto-cardiolipin Ab in lupus-prone mice, and 4) autoimmune cellular profiles:

quantification of leukocyte subpopulations changes and cytokine mRNA expressions about activation status in spleen. **Results:** In the first experiment, there were no significant differences among control, non-CpG ODN and mouse CpG ODN 10 μg groups: in non-specific immunotoxicological profiles (BW, spleen/BW ratio, and WBC, RBC, SGOT, and SGPT) and in evaluation profiles of glomerulonephritis (BUN, Cr, BUN/Cr ratio, renal histopathology and proteinuria). However, titer of anti-dsDNA and anti-cardiolipin Abs in mouse CpG ODN group rose three or eight-fold higher than in control group. Next in the second experiment, there were significant differences in spleen/BW ratio (control vs. mouse CpG ODN 50 μg treated group, $p<0.05$) and kidney/BW ratio (control vs. mouse CpG ODN 30 μg treated group, $p<0.05$) of nonspecific immunotoxicological profiles among groups. Hematological parameters of mouse CpG ODN-treated groups were within upper normal limit, whereas control group manifested anemia and thrombocytopenia in blood counts. Amount/titer of anti-dsDNA and anti-cardiolipin Abs in mouse CpG ODN group rose three or eight-fold higher than in control group, while all groups revealed increased BUN/Cr ratio and/or proteinuria, and diffuse proliferative glomerulonephritis, Class IV. **Conclusions:** Collectively, these results show that CpG ODN might be less immunotoxic or attenuating autoimmune indexes on lupus-prone NZB/NZW F1 female mice, clinically implying that CpG ODN-based immunotherapy might be justifiable in lupus-prone host.

Key words: CpG oligodeoxynucleotide; Immunotoxicity; SLE; NZB/NZW F1 mouse

Immunotoxicological effects of mouse CpG ODN in lupus-prone NZB/NZW F1 mice

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(Directed by Professor Soo-Ki Kim)

I. INTRODUCTION

1.1. CpG oligodeoxynucleotides

Unmethylated Cytosine-Guanine oligodeoxynucleotides (CpG ODNs) originate from DNA fragments of purified *Bacillus Calmette-Guerin* (BCG). It was first discovered by Tokunaga and his coworkers that BCG as well as BCG-derived DNA fragments had an innate antitumor activity (1). These bacterial DNA fragments or unmethylated CpG ODN motifs are prevalent in prokaryotes but not eukaryotes genomic DNAs (2, 3). Since the activity of CpG ODN was discovered by Krieg et al. (1995), it has been well established that CpG ODNs, artificially synthetic mimicry of natural bacterial DNA, modulate innate and adaptive immune response cells such as DC, NK, and B cells, and produce Th1-type proinflammatory cytokines, interferons, and chemokines (2, 4-10). CpG ODNs as pathogen associated molecular patterns (PAMPs) are directly recognized by pattern-recognition receptors (PRRs), Toll-like receptor 9 (TLR9). Specifically, TLR9 has been shown to be essential for the recognition of bacterial

DNA containing unmethylated CpG (2, 10-13). In parallel, TLR9 might be involved in pathogenesis of autoimmune disorders by way of sensing the chromatin structure (10, 14-16).

In general, CpG ODNs directly stimulate TLR9 expressed immune cells and indirectly activate TLR9-free immune cells, and induce a pattern of Th1-typed immune responses. As for the activation of innate immune cells such as dendritic cell (DC), macrophage cells, and NK cells, CpG ODNs activate directly DC or macrophage, and increase surface expression of MHC class II, ICAM-1, or the co-stimulatory molecules (CD40, CD54, CD80, and CD86), cytokine secretion (IL-6, IL-12, and TNF-α), IFN-α/β secretion, and chemokine production (IL-8, IP-10, and GM-CSF) (2, 4, 9). In particular, CpG ODNs indirectly stimulate NK cells to synthesize IFN-γ and to enforce lytic activity (2, 17, 18). As for the stimulation of adaptive immune cells, such as B and T cells, in B cells CpG ODNs activate mitogen, induce B cells to enter the G1 phase of the cell cycle, and stimulate increased level of costimulatory molecules such as MHC class II, CD40, CD54, CD80, and CD86 (2, 9). The CpG-triggered IL-6 and IL-10 respectively render B cells to secrete IgM and to reduce IL-12 secretion. Additionally, in mature B cells low dose of CpG DNA strongly augments B cell proliferation and antigen-specific Ig secretion or IL-6 secretion (2). As for T cells, CpG ODNs direct cytokine milieu toward Th-1 typed immune response through the activation of antigen presenting cells (APCs) such as DC, macrophages, or some B cells, thereby resulting in the activation of cytotoxic T lymphocytes (CTLs) (2).

CpG ODNs are classified as type D (known as CpG-A), K (known as CpG-B) and C. Each of them has different structural characteristics and immunomodulatory activity. First, type D (A) structure of ODN shows mixed phosphodiester or phosphorothioate backbone, and single CpG motif, CpG flanking region forms a panlindrome, and poly-G tail at 3'end. Type D (A) ODN facilitates antigen presenting cell (APC) maturation to be mediated by IFN-α and

preferentially stimulates pDCs to secrete IFN-α. Second, type K (B) ODN structure shows phosphorothioate backbone, multiple CpG motifs, and 5' motif most stimulatory. Type K (B) ODN modulates pDC maturation and TNF production, triggers B cell proliferation, and IgM and IL-6 production. Last, type C ODN structure shows phosphorothioate backbone, multiple CG motifs, TCG dimer at 5' end, and CpG motif embedded in a central palindrome. Type C ODN stimulates B cell to produce IgM and IL-6, and also activates pDCs to secrete IFN-α as combining characteristics of type D (A) and K (B) (2, 3, 19).

Since its discovery, CpG ODNs as vaccine adjuvant or immunotherapeutic have been widely used in preclinical and clinical fields (Table 1, 2, and 3). In particular, CpG-induced activation of innate and adaptive immunity confers protection or treatment against infection, allergy and cancer (2, 3, 19). Of these three intractable diseases, CpG ODNs have shown anti-infectivity against a variety of pathogen including bacteria (typical, atypical), virus, fungi, and tropical parasites. Anti-infective effects of CpG ODNs in pathogen challenge models were shown in Table 1. As for against cancer, CpG ODNs displayed potent anti-cancer effects in murine/rodent model including; tumor eradication, enhancing cancer immune surveillance, and preventing tumor development (Table 2). For human trial, CpG ODN based immunotherapy has been initiated alone or in combination with chemotherapy, radiotherapy, cell therapy, antibody therapy, and/or surgery (Table 3). As for against allergic disease, CpG ODNs have clearly shown protection or therapeutic efficacy by way of generating Th1-skewed immune response (19-23).

Despite the versatile applicability of CpG ODN, the safety and toxicity of CpG ODNs were partly reported but not completely established (Table 4). While CpG ODNs are in nature nucleotides, these chemical structures might provoke autoimmunity or hypersensitivity by excessive stimulating Th1 immunity (2, 3, 24, 25). However, these potential

immunotoxicological profiles and mechanisms of CpG ODNs remain unsettled. Moreover, emerging demand and clinical usage of CpG immunotherapy prompted me to investigate the immunotoxicological impact of CpG ODNs in lupus-prone mice.

Table1. Anti-infective effects of CpG ODNs in pathogen challenge models*

Species	Pathogen Class	Challenge	References
Mouse	Bacteria	*Listeria monocytogenes*	(26-30)
		Fransicella tularensis	(28, 30)
		Mycobacterium avium	(33)
		Mycobacterium tuberculosis	(38)
		Helicobacter pylori	(41)
		Burkholderia mallei	(43)
		Polymicrobial sepsis (peritonitis)	(45)
		Escherichia coli DH5{alpha} and *Escherichia coli* K1 strains	(47)
	Parasite /other	*Leishmania major*	(49, 50)
		Plasmodium yoelii	(52)
		Toxoplasma gondii	(56)
		Cryptococcus neoformans	(58, 59)
		Cryptococcus parvum	(60)
		Scrapie prion	(62)
	Virus	Respiratory syncytial virus	(31)
		Tacarbie arenavirus	(32)
		Herpes simplex virus	(34-37)
		Influenza	(39, 40)
		Orthopoxvirus	(42)
		Friend retrovirus	(44)
		Foot and mouth disease virus	(46)
		Lymphocytic choriomeningitis virus (LMCV)	(48)

Table1. Anti-infective effects of CpG ODNs in pathogen challenge models* (continued)

Species	Pathogen Class	Challenge	References
Goat	Bacteraia	*Escherichia coli*	(51)
Chicken	Bactria	*Escherichia coli*	(53-55)
		Salmonella Typhimurium	(57)
Guinea pig	Virus	Herpes simplex	(37)
Hamster	Virus	Punta Toro virus (PTV, Bunyaviridae, phlebovirus)	(61)
Macaques	Vrius	simian immunodeficiency virus (SIV) model	(63)

*This Table 1 was modified from Krieg, A. M. 2007. Antiinfective application of toll-like receptor 9 agonists. Proc Am Thorac Soc 4:289-94.

Table 2. Anti-cancer effects of CpG ODN in murine model

Tumor	References
Colon Ca with/without liver /peritoneum metastasis	(64-67)
Glioma	(68, 69)
Lymphoma / B cell lymphoma / acute lymphoblastic leukemia (ALL) / chronic lymphocytic leukemia B cells / acute myelogenous leukemia (AML)	(70-76)
Ovarian Ca	(77)
Breast Ca	(78, 79)
Melanoma	(71, 80, 81)
Bladder Ca	(82-85)
Pancreatic Ca	(86)

Table 3. CpG ODN 7909 and 1018 ISS clinical trial for human therapy

Type of CpG	Function	Goal of treatment	References
CpG 7909	Vaccine therapy as an adjuvant	Hepatitis B vacine	(87-91)
		Malaria / *Plasmodium falciparum* malaria vaccine	(92, 93)
		Pseudomonas aeruginosa ExoProtein A vaccine	(94)
		Fluarix Influenza vaccine	(95)
		CETP (Cholesteryl ester transfer protein: anti-atherosclerosis) vaccine	(96)
	Anti-cancer single or combination therapy	Non-small-cell lung cancer	(97)
		Melanoma with / without metastases	(98-101)
		Basal cell carcinoma	(101)
		Non-Hodgkin's lymphoma	(102, 103)
1018 ISS	Vaccine therapy as an adjuvant	Hepatitis B vaccine	(104)
	Anti-cancer single or combination therapy	Non-Hodgkin's lymphoma	(105)

Table 4. Toxicity of CpG ODN

Target organs	Disease / Manifestation	Animal model	Mechanism	References
Kidney	Glomerulonephritis SLE- like autoantibody symptoms	Normal mice, MRL and MRL $^{lpr/lpr}$ NZB × NZWF1 (B/WF1) mice HAF-induced glomerulonephritis	Immunoglobulin complex Prominent deposits of IgG_{2a} and C3, IgG_{2a} type anti-nuclear antibody in the blood. IL-6 B cell IL-12p40, B cell, auto-dsDNAAbs, DC IFN-α immune complex: role of TLR9-mediated expression of chemokines and chemokine receptors TLR9 activation on B cells, macrophage, and DCs	(106-109)
Liver	Hepatitis / ascites	Normal mice, Con-A induced hepatitis mouse model CRP-K^b×Des.TCR mice	IFNs and TNF-α Activated T cells and autoimmune damage	(110-112)
Spleen	Splenomegaly	Normal mice MRL and $MRL^{lpr/lpr}$	IFNs and TNF-α B cell IL-12p40, B cell, auto-dsDNAAbs, DC IFN-α	(108, 110)

Table 4. Toxicity of CpG ODN (continued)

Target organs	Disease / Manifestation	Animal model	Mechanism	References
Joint	Arthritis	LEW and LEW 1AV1 rat Normal mice LEW and Normal mice	Lymphoplasia and IL-1β & IFN-γ Macrophage and IL-12 IFN-γ, the osteoclast differentiation factor, and receptor activator of NF-kappaB ligand (RANKL). systemic joint inflammation by induction of inflammatory Th1 immune responses: macrophages, dendritic cells, and osteoclast precursors in the bone marrow	(113-115)
Colon	Colitis	Dextran-sulfate-sodium (DSS)-induced colitis mouse model	IFN-γ, TNF, and IL-12	(116)
Hematology	Leukocytopenia	Normal mice	Follicular DC and B lymphocyte suppression Bone marrow suppression	(110)
	Anemia	Normal mice (High dose CpG treated only)	Erythropoietic suppression by pro-inflammatory cytokines (IL-12, TNF-α, IFN-α, IL-1α,β)	(117), unpublished our data
	Septic shock	Normal mice	TNF-α	(118)
Survive	Opportunity infection	*Candida albicans* treated normal mice	IL-2	(119)

1.2. Immunotoxicology

Immunotoxicology is the subdisciplinary toxicology dealing with purposeful or inadvertent changes or effects on the immune system induced by drugs, foods or environmental chemicals (120). Immunotoxicology covers immunosuppression, autoimmunity or chronic inflammation, and immunomodulation. Immunosupression decreases humoral or cellular immunity needed by the host to defend itself against infectious agents or cancer; autoimmunity or chronic inflammation cause tissue damage; immunomodulation increases the frequency of immediate allergic reactions and/or decreases the frequency or intensity delayed hypersensitivity reactions (121).

There are three representative immunotoxicity guidelines as follows: the European Agency for the Evaluation of Medicine / Committee on Proprietary Medicinal Product (EMEA/CPMP), the Ministry of Health, Labour and Welfare (MHLW) in Japan, the U.S. Food and Drug Administration (FDA) (121-123). According to the summary of these guidelines, immunotoxicological test comprises two parameters: non-functional and functional parameters. First, non-functional parameters consist of routine hematology with differential cell counting and clinical chemistry, BW, lymphoid organ (thymus, spleen, local and distinct lymph nodes) weights, histopathology of lymphoid organ, and bone marrow cellularity. Second, functional parameters as a screening of functional immunologic effects or evaluation of potential immunotoxicological risks consist of lymphocyte subsets, NK cell function, T cell function, serum titeration of IgM, IgG, IgA, and IgE, mitogen response, macrophage function, host-resistance included infection model, endotoxin hypersensitivity, and autoimmune models (121, 123).

CpG ODN as an immunostimulatory drug requires gauging potential immunotoxicological hazard such as autoimmunity, owing to uprising argument of CpG ODN usage. Unfortunately, all of the guidelines have not strict rules on autoimmunity testing for immunotherapeutics (121-123). In fact, while there were well documented in the mechanisms or efficacies of CpG ODN, there are poorly documented about immunotoxicity of CpG ODN based on autoimmunity test. Previous studies have partially suggested that potential immunologic hazards of CpG ODN or DNA might exacerbate the progress of autoimmune disease in normal or lupus-prone mouse model (106-109). However, systemic analyses about immunotoxicity of CpG ODN have been not initiated. Thus, autoimmunity test-guideline of CpG ODNs based on SLE disease-specific manifestations and immunotoxicological guidelines was made. Next, to test *in vitro* as well as *in vivo* immunotoxicity of CpG ODN, spontaneous lupus model, NZB/NZW F1 was adopted. Thereafter, their autoimmune parameters were analysed (Table 5). Here, autoimmune parameters encompass 1) nonspecific immunotoxicological profiles: BW, organ weight, and routine hematology and/or serology, 2) specific autoimmune organ profiles: lupus nephritis and proteinuria level, 3) autoimmune seromarker / autoantibody marker: titer of auto-dsDNA Ab and auto-cardiolipin Ab in lupus-prone mice, and 4) autoimmune cellular profiles: quantification of leukocyte subpopulations changes and cytokine mRNA expressions about activation status in spleen.

Table 5. Measurable parameters of autoimmunity

Parameters	Procedures
Non-functional	- Routine hematology including differential cell counting - Serum IgM, G, A, and E - Lymphoid organ weights with including kidney - Histopathology of immune organs - Bone marrow cellularity - Analysis of lymphocyte subpopulations in spleen
Host-resistance	Autoimmune models: NZB/NZW F1
	- Urine test: proteinuia - BUN, Cr, and BUN/Cr - Histopatholgy of Kideny: H&E, EM, six IFs - Autoantibody in serum: anit-dsDNA, anti-cardiolipin - Serum IgG_1 and IgG_{2a} - lupus-related cytokine expression: IL2, IFN-γ, TNF-α, IL-10

1.3. Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a multisystemic, tissue destructive disease that is caused by autoantibody production and complement fixing immune complex deposition into parenchymal tissue. The main immunological abnormalities of SLE are B cell hyperactivity, autoantiboy production and immune complex deposition in vital organs. According to organ-specific targets, SLE has a wide spectrum of clinical presentations. Clinical manifestations of SLE consist of as follows: malar rash, discoid rash, photosensitivity, oral ulcers, arthritis, serositis, renal disorder, neurologic disorder, hematologic disorder, and immunologic disorder in human.

Likewise in mucocutaneous system, one of the most commonly affected, malar rash is a fixed flat or raised erythema and discoid rash is erythematosus raised pathches with adherent keratotic scaling, follicular plugging, or atrophic scarring in older lesions. Mucosal lesions are also part of the clinical spectrum of SLE and can affect the mouth, nose, and anogenital area as an ulcer. In musculoskeletal system, painful joints are the most common presenting symptom of SLE with nonerosive arthritis involving two or more peripheral joints, tenderness, swelling or effusion. Also generalized myalgia or muscle weakness and myositis, frequently involving the deltoids and quadriceps, can be accompanying features of disease flares. In renal system, the kidney is regarded as a signature organ affected by SLE. Importantly, myriad of studies about the prognosis in SLE have identified lupus nephritis as an important predictor of disease severity/activity. Classification of lupus nephritis by World Health Organization ranges from Class I to V, which should be assessed by histology and location of immune complexes with clinical clues: sediment, proteinuria, serum creatine, blood pressure, anti-dsDNA, and C3/C4. In nervous system, approximately two thirds of patients with SLE have

neuropsychiatric manifestations, seizures or psychosis. Additionally in cardiovascular and pulmonary system, there are the most common signs and symptoms as follows: serositis, endo- or pericarditis, chest pain, atherosclerosis, dyspnea, pleural effusion, pleuritis, pneumonitis, pulmonary hemorrhage or embolism, pulmonary hypertension, and shrinking lung. Clinically important hematologic or immunologic disorders are “hemolytic anemia with reticulocytosis, leucopenia, lymphopenia and thrombocytopenia”, autoimmune related antibodies, “anti-DNA, anti-SM, or anti-cardiolipin”, and also complement, “C3 and C4” (124-126).

All autoimmune diseases do not develop without interplay of genetic factors, infection, and other environmental factors. These trigger factors turn from normal autoimmunity to autoimmune disease that breaks down immune response and tolerance. CpG ODN as a potent stimulator mimicking bacterial DNA might tend to change immune status in lupus-prone host to worsen or progress autoimmune condition. In fact, some preclinical reports have suggested that repeated CpG ODN injection might aggravate autoimmune disease such as lupus nephritis (24, 107-109, 127).

To resolve this tissue, I have searched for the most suitable lupus mouse model to gauge the immunotoxicological impacts of CpG ODN. There are two types: spontaneous and induced lupus nephritis model in mice. For autoimmunity test, spontaneous mouse model would be more precise, reproducible and suitable because provocative drug might interrupt immunotoxicity of the CpG ODN in the induced lupus model. Among spontaneous mouse stains, NZB/NZW F1 mouse or MRL-lpr/lpr mouse develops pathology resembling human lupus. These two models have been chiefly used in the study of the pathogenesis of autoimmunity and preclinical trial of immunosuppressive drugs (121, 123). NZB/NZW F1 mouse is more natural model than MRL-lpr/lpr mouse because MRL-lpr/lpr mouse turns gene

knocked-out. The lpr mutation leads to the absence of Fas, a cell-surface molecule that triggers apoptosis in lymphocytes. Additionally, New Zealand strains have an MHC-linked deficiency in the expression of the pro-inflammatory cytokine TNF-α (125). The characteristics of NZB/NZW F1 mice were listed in Table 6. Based on this rationale, I selected NZB/NZW F1 female mouse to test the possible autoimmunity of CpG ODNs.

SLE is a prototypic autoimmune disease associated with the production of antibodies to self DNA. Since unmethylated CpG ODN could function as vaccine adjuvant, CpG ODN theoretically might induce polyclonal B cell activation, autoantibodies against self DNAs or protein, thereby forming immune complexes. These immune complexes were finally deposited in vital organs such as kidney, in the end leading to damage or malfunction. Thus, to address this, I investigated whether optimal or escalated dosing of CpG ODN treatment would affect immunotoxicological parameters reflecting autoimmunity status in NZB/NZW F1 mice.

Table 6. Characteristics of NZB/NZW F1 female mice*

Part	Characteristics
Clinical symptom	Live: a mean of 280 days Proteinuria#: 333 mg/100 ml level to detect heavy proteinuria (25% about 7 months, 50% about 8~9months, 90% about over 12 months) Death: caused by immune glomerulonephritis Fifty percent mortality: by 8 months
Histologic features	Glomerulonephritis with proliferative: changing in mesangial and endothelial cells of glomeruli, capillary basement membrane thickening, and chronic obliterative changes; mononuclear cell infiltrates in interstitium Glomerular immune deposits: IgG (predominatly IgG_{2a}) and C3 (similar deposits in tubular basement membrane and interstitium) Thymic cortical atrophy: by 6 months of age Myocardial infarcts with hyaline thickening of small arteries Mild lymph node hyperplasia and splenomegaly
Autoantibodies	IgG anti-dsDNA (also binds ssDNA) (enriched in IgG_{2a} and $_{2b}$) ANA and LE cells in all IgG antibodies: binding chromatin, nucleosomes, and phospholipids Antithymocyte Renal eluate: containing IgG anti-dsDNA concentrated 25 to 30 times greater than in serum; IgG_{2a} isotype: usually dominant Modest elevations of circulating immune complexes Low serum complement levels: by 6 months of age

Table 6. Characteristics of NZB/NZW F1 female mice* (continued)

Part	Characteristics
Immune abnormalities	Polyclonal B cell activation B cells: resistant to tolerance to some antigens Strict dependence on T cell: helping for formation of pathogenic IgG anti-DNA ($CD4^+CD8^-$ and $CD4^-CD8^-$ α/β TCR cells as well as $CD4^-CD8^-$ γδ TCR cells, can provide help) IgG repertorie: restriction of B cell clonality in the IgG anti-DNA response Thymic epithelial atrophy: by 6 months of age; medullary hyperplasia; effect to thymectomy on disease varies Clearance of immune complexes by Fc-and complement-mediated mechanisms: defective Disease and autoantibody production: sensitive to sex hormone influences
Genetics	The expression of high-titer IgG anti-dsDNA: requiring heterozygosity at MHC, namely $H\text{-}2^{d/z}$

*This Table was adopted from "Chapter 18. Table 18-3. Charateristicis of NZB/NZW F1 Mice" in Dubois' Lupus Erythematosus 7th edition, Lippincott Williams & Wilkins.
#Proteinuira was supplemented from Knight, J. G. and D. D. Adams. 1978. Three genes for lupus nephritis in NZB × NZW mice. J Exp Med 147:1653-1660.

II. PURPOSE

Unmethylated CpG ODNs mimicking bacterial DNA fragments are directly sensed by TLR9 (2, 10-13). Well-reportedly, CpG ODNs coordinate innate and adaptive immune response cells such as lymphocytes, DC, NK cells and macrophages, and induce Th1-type proinflammatory cytokines, interferons, and chemokines release (2, 4-10). In that context, CpG ODN as vaccine adjuvant or immunotherapeutic widely confers protection or treatment against infection, allergy and cancer in preclinical and clinical fields (2, 3, 19).

Despite the versatile applicability of CpG ODN, the safety and toxicity of CpG ODNs were veiled. While CpG ODNs are in nature nucleotides; these chemical structures might provoke autoimmunity or hypersensitivity through overdue Th1 polarized immunity (2, 3, 24, 25). Besides, since unmethylated CpG ODN could function as a vaccine adjuvant, CpG ODN theoretically might induce polyclonal B cell activation, autoantibody against self DNAs or protein, thereby forming immune complexes. These immune complexes might be deposited in vital organs such as kidney, consequently leading to damage or malfunction.

However, these potential immunotoxicological profiles and mechanisms of CpG ODNs remain unsettled such as autoimmunity, owing to uprising argument of CpG ODN usage. Unfortunately, all of the guidelines have not strict rules on autoimmunity testing for immunotherapeutics (121-123). Towrad this, first, autoimmunity test-guideline of CpG ODNs based on SLE disease-specific manifestations and immunotoxicological guidelines was made. Next, to test in vitro as well as in vivo immunotoxicity of CpG ODN, spontaneous lupus model, NZB/NZW F1 was adopted. Thus I investigated whether optimal or escalated dosing of CpG ODN treatment would affect immunotoxicological parameters reflecting autoimmunity status in NZB/NZW F1 female mice.

Here, autoimmune parameters encompass 1) nonspecific immunotoxicological profiles: BW, organ weight, and routine hematology and/or serology, 2) specific autoimmune organ profiles: lupus nephritis and proteinuria level, 3) autoimmune serologic marker / autoantibody marker: titer of auto-dsDNA Ab and auto-cardiolipin Ab in lupus-prone mice, and 4) autoimmune cellular profiles: quantification of leukocyte subpopulations changes and cytokine mRNA expressions about activation status in spleen.

III. MATERIALS AND METHODS

3.1. Experimental protocols

3.1.1. The 1st experimental protocol: optimal dose treatment of mouse CpG ODN for 4 times weekly

NZB/NZW F1 female mice were divided into 3 treatment groups of six animals each. Control group received the vehicle saline alone, non-CpG group received the non-CpG ODN (1982) 10 μg per mouse, and mouse CpG group received the mouse CpG ODN (1826) 10 μg per mouse. Animals were intraperitoneally administered at 12, 13, 14, and 16 weeks of age thereafter weighed and examined as described below. Mice of each group were sacrificed with neck dislocation and their organs were removed aseptically 10~12 days after the last treatment.

3.1.2. The 2nd experimental protocol: escalated dose treatment of mouse CpG ODN for 8 times weekly and 12 weeks observation

NZB/NZW F1 female mice were divided into 4 treatment groups of three or six animals each. Control group received the vehicle alone, the other experimental groups received the mouse CpG ODN (1826) 10 μg, 30 μg, or 50 μg each mouse. Animals were intraperitoneally administered at 12, 13, 14, 15, 16, 17, 18, and 19 weeks of age and then observed for 12 weeks. At each time point mice were weighed and examined as follows. Mice of each group were sacrificed with neck dislocation and their organs were removed aseptically 4~12 days after the last treatment.

3.2. CpG oligodeoxynucleotides

The CpG ODNs were completely phosphorothioate-modified. These were provided by MWG-Biotech AG (Ebersberg, Germany). The sequences used were 1982 CpG ODN (non-CpG motif): 5' TCCAGGACTTCTCTCAGGTT 3', 1826 CpG ODN (mouse motif): 5' TCCATGACGTTCCTGACGTT 3'. All CpG ODNs contained <0.1 EU/ml of endotoxin as determined by the Limulus assay (Bio-Whittaker, USA).

3.3. Mice

NZB/NZW F1 female (10~11weeks old) and C3H mice were purchased from JungAng Experimental Laboratories (Seoul, Korea). The animals were acclimated in a controlled-environment animal room (temperature, 21±3℃; humidity 30~70%; photoperiod, 12-h light/dark cycle) and were fed on commercial pellets and tap water. Procedures involving the care and use of animals in these studies were reviewed and permitted by the Graduate School of Yonsei Wonju Medical University.

3.4. Hematology and serum chemistry

Blood samples were taken from the orbital of mice into Mricrotainer Brand tubes (BD, USA) for whole blood and empty tube for serum examinations, respectively, under ether anesthesia, and then the animals were euthanized by exsanguinations. The blood was measured for the counts of red blood cell (RBC), white blood cell (WBC), differential WBC [lymphocytes (LY), neutrophils (NE), monocytes (MO), eosionophils (EO), and basophils (BA); each cell count was calculated from its ratio and the WBC count] and platelets (PLT), hemoglobin concentration (Hb), hematocrit value (Hct), and (MCV) with HEMAVET 950FS

(Drew scientific Inc., USA). Sera were separated and subjected to the measurement of aspartate aminotransperase (AST), alanine aminotrasperase (ALT), blood urea nitrogen (BUN), and creatinine (Cr) with DRI-CHEM 3500i (FujiFilm, Japan).

3.5. Auto-dsDNA and -cardiolipin antibody levels in serum

I collected weekly blood from the retro-orbital venous plexus under general anesthesia with inhaled ether, and centrifuged the blood sample to obtain each serum from grouped NZB/NZW F1 mice. The brief measurement procedures were as follows: for anti-dsDNA Ab or anti-cardiolin (CL) Ab, ELISA 96 well microtiter plates were coated overnight at 4℃ with calf thymus dsDNA (Sigma, USA) or Bovine Heart cardiolipin sodium salt (Sigma aldrich, USA) antigen at a concentration of 10 ㎍/ml; 200 ㎕ blocking buffer (5% bovine serum albumin-and 0.05% Tween 20-containing phosphate buffered saline) was placed in each well, and incubated for 1h at 37℃; collected serum diluted 3,000 or 10,000 times with diluents (0.5% bovine serum albumin-and 0.05% Tween 20-containing phosphate buffered saline) for IgG was added at 100 ㎕/well, and incubated for 2h at 37℃; alkaline phosphate-conjugated goat anti-mouse IgG (SouthernBiotech, USA) diluted 3,000 and 10,000 times, respectively, with the diluents was added at 100 ㎕/well, and incubated for 2h at 37℃; p-nitropheylphosphate liquid substrate solution (Sigma, USA) was added at 100 ㎕/well, and incubated for 15min at room temperature. The optical density of the test solution was read at 405 nm, and the absorbance value was used.

3.6. IgG1 and IgG2a levels in serum

The amounts of IgG1 and IgG2a level in sera of pre-and post-fourth treatment were measured by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Koma biotech, Korea). Developed color reaction was measured as OD unit at 450 nm on Emax, ELISA reader (Molecular Devices, USA). The each concentration of IgG1 and IgG2a in μg/ml by kit's standard curve was expressed. The experiments were performed in triplicate for each concentration of sera.

3.7. Leukocyte subpopulations from spleen

Splenocytes from each group NZB/NZW F1 mice were obtained aseptically and purified using 0.7 μm mesh (BD Falcon, USA). A direct immunofluorescence technique was used to measure FITC- or PE-conjugated CD45R/B220+ cells, CD3+ cells, CD4+ cells, CD8+ cells, and CD4+CD154+ cells (BD Pharmigen, USA) or CD11c+ cells, CD80+ cells, CD86+ cells, and I-A/I-E+ cells (eBioscience, USA) in spleen of NZB/NZW F1 mice. Briefly, 1×10^6 cells were respectively stained with the appropriate monoclonal antibodies on ice for 30 minutes, and then cells were washed twice in Hanks Balanced Salt solution with 1% BSA / 0.1% sodium aside. Subsequently, they were washed twice and fixed in 500 $\mu\ell$ PBS / 1% paraformaldehyde (Sigma-Aldrich, USA). Data on 50,000 events were acquired and processed using a Cytomics FC 500 (Beckman Coulter, Germany).

3.8. Proteinuria

I collected weekly fresh spot urine samples from all NZB/NZW F1 mice of each treated group as protocols. To quantify protein concentration in fresh urine, I used Bradford assay in every week. Shortly, I put each 10 $\mu\ell$ of spot urine and 2 fold diluted solution 10 $\mu\ell$ of 10~40 mg/ml albumin solution to draw standard curve of protein concentration in 96 well plate. Bradford solution (BioRad, USA) diluted 1:5 in distilled water and transfer 200 $\mu\ell$ in each well. Developed color reaction was measured as OD unit at 595 nm on Emax, ELISA reader (Molecular Devices, USA). The concentration of proteinuria in mg/ml by standard curve was expressed. The experiments were performed in triplicate for each concentration of all spot urine.

3.9. Cytokine mRNA expressions in splenocytes

3.9.1. Separation of splenocytes

After sacrificing all NZB/NZW F1 mice treated as the first or second protocol, single cell suspensions were prepared from all mice in experiment. Splenocytes were isolated aseptically from each mouse, and harvested in RPMI1640 complete medium with 1% penicillin-streptomycin-amphotericin B and 10% fetal bovine serum (FBS). Before cell pellets were resuspended and maintained in 2 ml RPMI1640 medium on ice, all RBCs were removed by 0.83% NH_4Cl RBC lysis sloution.

3.9.2. RNA extraction from samples and preparation of cDNA

Total RNA was isolated using TRIzol reagent (Invitrogen Life Technologies Inc., USA) as described in the manual, and the amount of RNA obtained was determined by

spectrophotometry. A total 1 ㎍ of RNA was mixed with 500 ㎍/ml oligo $d(T)_{15}$ Primer, 200 U/ml of MMLV reverse transcriptase, 10mM of the dNTP, 40U/㎕ of RNase inhibitor in M-MLV RT 5× buffer (Promega, USA) was added in a final volume of 25 ㎕. The mixture was incubated at 42℃ for 30 min and the reaction was terminated at 95℃ for 5 min. RT-PCR was performed with MJ Mini personal thermal cycler (BioRad, USA).

3.9.3. Semiquantitative RT-PCR

For PCR, 25 ㎕ of PCR mixture was prepared in 2 ㎕ of cDNA supplemented with 10mM Tris-Hcl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 5 ㎛ M dNTP, 1U Taq DNA polymerase (Takara, Japan) and 20 pmol of forward and reverse primers (COSMO, Korea). The sequences and annealing temperatures of each primer sets were described in Table 7. After RT-PCR, PCR products were analyzed by electrophoresis in a 1.2% agarose gel contain with ethidium bromide. Amplified DNAs were quantified using the FlourS-MultiImager system (Biorad, Germany).

Table 7. Sequences of PCR primers

Primers of mouse	Sequence (5′ to 3′)	PCR products (bp)
β−actin	TGG AAT CCT GTG GCA TCC ATG AAA C TAA AAC GCA GCT CAG TAA CAG TCC G	348
IL-2	GCT CCT GAG CAG GAT GGA GAA TTA CAG GAG AGC CTT ATG TGT TGT AAG CAG GAG G	375
IL-10	AGA AAT CAA GGA GCA TTT GA CTG CAG GTG TTT TAG CTT TT	251
IFN-ɣ	TTG GAT ATC TGG AGG AAC TG CCT CAA ACT TGG CAA TAC TC	281
TNF-α	GAG TGA CAA GCC TGT AGC CCA TGT TGT AGC A GCA ATG ATC CCA AAG TAG ACC TGC CCA GAC	307

3.10. Histopathology

3.10.1. Light microscopy

Renal tissue was fixed in 10% neutral buffered formaldehyde, and 3 ㎛, paraffin-embeded sections were stained with hematoxylin and eosin (H&E) and Priodacid-Schiff stain (PAS). The severity of the glomerulonephritis was graded by world helath oranization (WHO) classification of lupus nephritis by pathologist (Table 8).

Table 8. WHO Classification of Lupus Nephritis*

Class	Pattern	Site of immune complex deposition
I	Normal	None
II	Mesangial	Mesangial only
III	Focal and segmental proliferative	Mesangial, subendothelial
IV	Diffuse proliferative	Medangial, subendothelial
V	Membranous	Subepithelial intramembranous

*This Table was adopted from "Chapter 17. Table17B-1. World Health Organization Classification of Lupus Nephritis" Klippel, J. H. 2001. Primer on the rheumatic diseases. Arthritis Foundation, Atlanta, Ga.

3.10.2. Immunofluorescent stain

Each right fresh renal tissue was frozen in OCT compound in cryomolds and 2~3 ㎛ thick frozen sections were made by cryostat and fixed in ice cold acetone for 10 minutes, air dry for 10 minutes and wash three times with PBC solution for 10 minutes, In Brief, for detection of IgG, IgM, IgA, C1q, C3, and fibrinogen deposits (n=4) in glomeruli, each section was incubated with FITC-labelled rabbit anti-human/mouse IgG, IgM, IgA, C1q, C3, and fibrinogen antibody (Dako, USA).

3.10.3. Electron microscopy

For ultrastructural studies, the fresh renal tissue was immediately treated with 3% glutaraldehyde at 4℃for 24 hours. These samples were then rinsed with 0.1M sodium cacodylate buffer and post-fixed with 1% osmium tetroxide in the same buffer for 2 hours. After rinsing with 0.1M cacodylate buffer, they were dehydrated for 15 minute periods in increasing concentrations of ethanol (70, 80, 80, 95 and 100% v/v) and exchanged through propylene oxide, and embedded in a mixture of epoxy resin. Ultrathin sections were cut with a diamond knife on an ULTRACUT E ultramicrotome (Reichert-Jung, Austria) and were stained with 1% uranyl acetate for 14minutes, followed by a lead staining reagent for 3 minutes. The sections were examined with a transmission electron microscope JEM 1200 EX II (JEOL, Japan).

3.11. Statistical analysis

All of the data were expressed as mean±SD and analyzed using PRIZM 4 software (GraphPad Software, USA). I used the Kruskal-Wallis for differences over three groups, Mann-Whitney U test for inter-group differences, and spearman correlation to identify the progress of SLE disease in all mice parameters. $P < 0.05$ indicated statistical significance.

IV. RESULTS

4.1. The 1st Experiment results: optimal dosing of CpG ODN treatment

The first experiment was designed to investigate the immunotoxicological impact in lupus-prone NZB/NZW F1 received with optimal dose (10 μg) of CpG ODN for 4 weeks. In clinical trials, CpG ODNs were used at dose 0.0025~0.64 mg/kg for the treatment of cancer or other disease such as allergy and infection (102, 105, 128). This dosing corresponds to about 10 μg in mice. Based on this notion, probing immunotoxicity of CpG ODN with optimal dose and schedule in lupus-prone mice is essential owing to scanty evidences and, being worth predicting immunotoxicity of CpG-based immunotherapy in human. To the end, I examined the immunotoxicological profiles in NZB/NZW F1 received with optimal dose (10 μg) of CpG ODN.

4.1.1. Body weight and Spleen organ weight ratio

To determine the immunotoxicological effects in NZB/NZW F1, following CpG ODN injection, first I assessed body and organ weights of female lupus mice treated with non-CpG ODN, mouse CpG ODN, or saline only for control group. Body weight (BW) every week was measured. Spleen was weighed after sacrificed and isolated aseptically. BW (Fig. 1A) and spleen/BW ratio (Fig. 1B) were not different among three groups: control, non-CpG, and mouse CpG. After CpG ODN injection for 4 weeks, spleen was not enlarged.

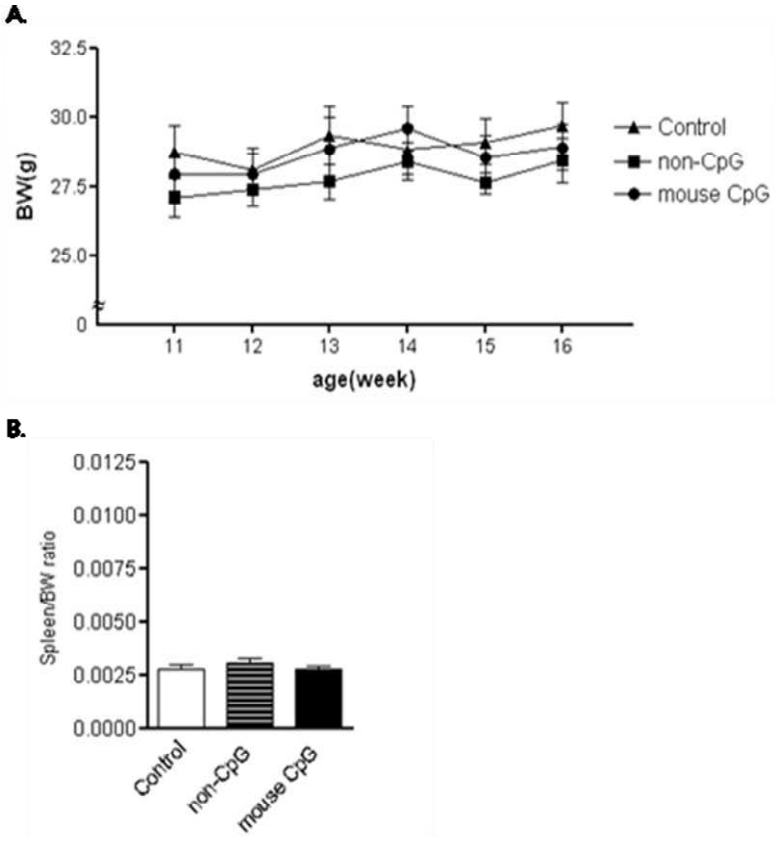


Figure 1. BW (A) and spleen/BW ratio (B) in optimal dose of CpG ODN-treated NZB/NZW F1 mice. After sacrificed each mouse was measured spleen organ weight. Data were shown as mean±SD and each group was an individual measurement (n=18).

4.1.2. Hematological parameters

To investigate hematopoietic impact of CpG ODN in lupus-prone mouse model, I performed WBC with differential counts, erythrocyte counts, and platelet counts for hematology function. Simultaneously in serum biochemical analyzes, I measured BUN, Cr, BUN/Cr ratio, SGOT, and SGPT for kidney and liver function.

In mouse CpG ODN (10μg) group, the counts of WBC, neutrophil (NE), lymphocytes (LY), monocytes (MO), eosinophil (EO), and basophil (BA) were slightly decreased, whereas lymphocyte percent of leukocytes was increased. There was no significantly different lymphocyte fraction of leukocytes among groups (Table 9).

In erythrocyte and platelet counts of mouse CpG ODN (10μg) group, there were higher hematocrit and lower MCH and MCHC than within normal range of control untreated mice. Increased hematocrit might indicate a decrease in total volume of body fluid due to relatively increased RBC count. Mouse CpG (10μg) group showed higher RBC count than normal range (Table 10). Platelet count and mean platelet volume, a measurement of the average size of platelets found in blood, were within normal range (Table 11).

In BUN, Cr, and BUN/Cr ratio for renal function test, BUN and BUN/Cr ratio were within normal range, but Cr of mice in all three groups were below 0.3 mg/dl, indicating that renal function was not aggravated (Table 12).

For liver function test, I selected serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT). All NZB/NZW F1 mice had a high SGOT value basically (Table 13).

Table 9. Leukocyte counts in optimal dose of CpG ODN-treated NZB/NZW F1 mice

	Normal range	Control (n=6)	Non-CpG (n=6)	Mouse CpG (n=6)	X^2	p
		MEAN(SD)				
WBC (K/$\mu\ell$)	1.8~ 10.7	5.84(0.741)	6.66(2.011)	4.93(0.889)	5.485	0.064
NE (K/$\mu\ell$)	0.1~ 2.4	1.38(0.322)	1.59(0.560)	1.00(0.191)	5.626	0.060
LY (K/$\mu\ell$)	0.9~ 9.3	3.94(0.425)	4.63(1.411)	3.57(0.750)	4.082	0.130
MO (K/$\mu\ell$)	0.0~ 0.4	0.32(0.197)	0.31(0.147)	0.22(0.054)	1.139	0.566
EO (K/$\mu\ell$)	0.0~ 0.2	0.15(0.119)	0.10(0.034)	0.10(0.052)	0.154	0.926
BA (K/$\mu\ell$)	0.0~ 0.2	0.05(0.033)	0.03(0.018)	0.04(0.026)	0.908	0.635
NE (%)	6.6~38.9	23.45(3.338)	23.31(3.441)	20.31(1.868)	3.520	0.172
LY (%)	55.8~91.6	67.78(6.228)	69.96(4.606)	72.09(2.796)	1.684	0.431
MO (%)	0.0~ 7.5	5.37(3.075)	4.67(1.612)	4.60(1.536)	0.012	0.994
EO (%)	0.0~ 3.9	2.57(1.864)	1.53(0.372)	2.17(1.159)	1.977	0.372
BA (%)	0.0~ 2.0	0.84(0.520)	0.53(0.210)	0.84(0.606)	1.276	0.528

Table 10. Erythrocyte counts in optimal dose of CpG ODN-treated NZB/NZW F1 mice

	Normal range	Control (n=6)	Non-CpG (n=6)	Mouse CpG (n=6)	X^2	p
		MEAN(SD)				
RBC (M/$\mu\ell$)	6.4~ 9.4	8.96(1.464)	9.25(0.590)	10.55(2.353)	1.509	0.470
Hb (g/dl)	11.0~15.1	12.12(1.839)	12.28(1.042)	11.95(1.100)	0.390	0.823
HCT (%)	35.1~45.4	48.22(7.787)	50.47(2.590)	56.13(11.513)	1.847	0.397
MCV (fL)	45.4~60.3	53.82(1.393)	54.62(1.162)	53.38(1.619)	2.380	0.304
MCH (pg)	14.1~19.3	13.78(3.099)	13.33(1.603)	11.58(1.432)	5.522	0.630
MCHC (g/dl)	30.2~34.2	25.53(5.161)	24.38(2.420)	21.68(2.678)	3.528	0.171
RDW (%)	12.4~27.0	18.07(0.753)	17.90(0.632)	19.25(1.392)	4.882	0.087

Table 11. Platelet counts in optimal dose of CpG ODN-treated NZB/NZW F1 mice

	Normal range	Control (n=6)	Non-CpG (n=6)	Mouse CpG (n=6)	X^2	p
		MEAN(SD)				
PLT (K/$\mu\ell$)	592~2972	650.83(166.150)	560.67(236.873)	657.33(189.908)	0.641	0.726
MPV (fL)	5.0~20.0	4.97(0.280)	5.10(0.424)	5.75(2.085)	0.063	0.969

Table 12. Serum renal function tests in optimal dose of CpG ODN-treated NZB/NZW F1 mice.

	Normal range	Control (n=6)	Non-CpG (n=6)	Mouse CpG (n=6)	X^2	p
		MEAN(SD)				
BUN (mg/dl)	9.2~29.2	18.68(3.191)	20.26(6.037)	18.67(4.348)	0.456	0.796
Cr (mg/dl)	0.4~ 1.4	0.30(0.000)	0.30(0.000)	0.30(0.000)	0.000	1.000
BUN/Cr ratio	6.57~73.0	62.28(10.636)	68.72(20.125)	62.22(14.492)	0.456	0.796

Table 13. Serum liver function tests in optimal dose of CpG ODN-treated NZB/NZW F1 mice.

	Normal range	Control (n=6)	Non-CpG (n=6)	Mouse CpG (n=6)	X^2	p
		MEAN(SD)				
SGOT (mg/dl)	17~44	75.00(13.624)	74.67(18.662)	78.00(23.108)	0.026	0.987
SGPT (mg/dl)	17~78	19.67(2.338)	19.00(2.449)	21.33(1.966)	3.769	0.152

4.1.3. Proteinuria

To determine the renotoxic effect in NZB/NZW F1, following CpG ODNs injection, I measured weekly spot urine to test protein level. Ten mg/ml concentration of albumin solution was used to draw a standard curve in ELISA (Fig. 2). Proteinuria is a sensitive marker to identify glomerulonephritis in NZB/NZW F1 mice. If protein level in urine rises more than 3.33 mg/ml, it refers to serious renal damage. All three groups revealed low protein level below 0.75 mg/ml.

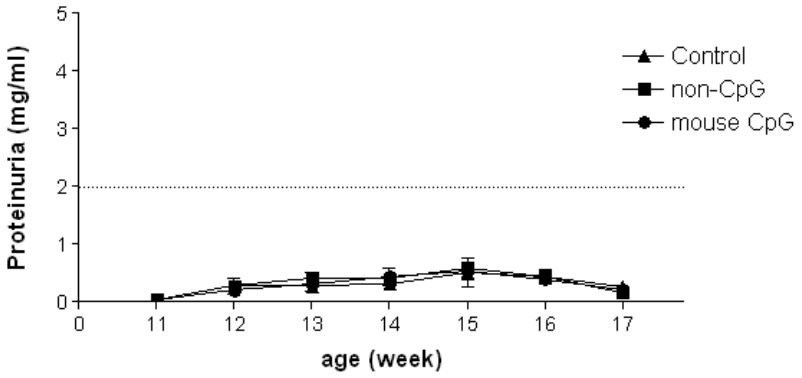


Figure 2. Proteinuria test in optimal dose of CpG ODN-treated NZB/NZW F1 mice. At each time point, spot urine collected from each mouse was tested for the presence of protein in urine by Bradford assay. Data were shown as mean±SD and each group is an individual measurement (n=18).

4.1.4. Renal histopathology

To verify glomerular changes following CpG ODN injction, NZB/NZW F1 mice were treated with saline or mouse CpG ODN (10 μg) intraperitoneally for 4 weeks, and examined for H&E and immunofluorescent (IF) stains. On the light microscopic findings, the numbers and sizes of glomeruli were within normal range without cellular proliferation or leukocytes infiltration in both control and mouse CpG ODN group. Their capillary loops were even and thin and those lumens were patent. The tubules, blood vessels, and interstitium were unremarkable. The immunofluorescent findings revealed nonspecific IgM and C1q deposits in the mesangium of control group and no deposit in the mouse CpG ODN group (Fig. 3 and 4). According to the WHO classification of lupus nephritis, these findings were consistent with Class I.

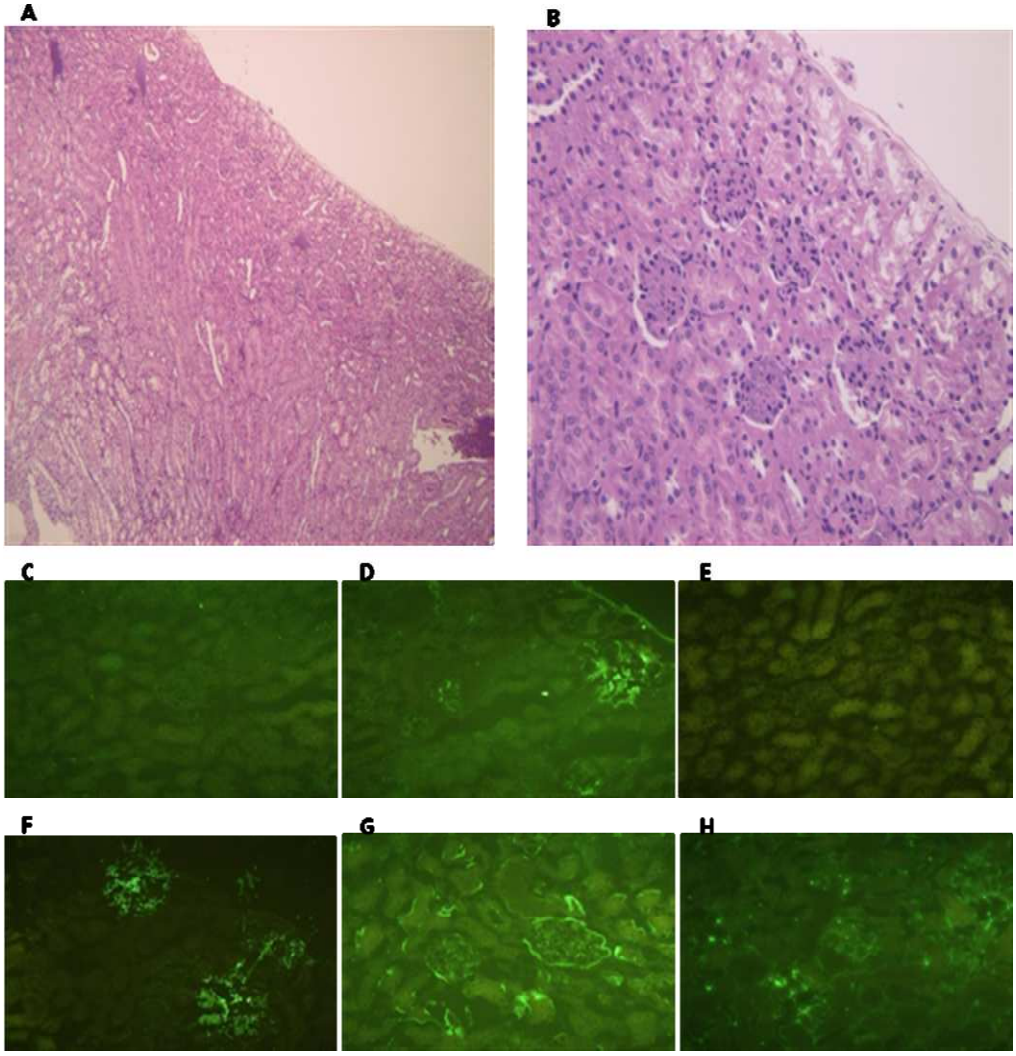


Figure 3. Renal histology of control group (16-week-old NZB/NZW F1 mouse) showing normal findings. Cortical sections from representative mouse of control group were stained with H&E (A and B), IF for IgG (C), IgM (D), IgA (E), C1q (F) C3 (G), and fibrinogen (H). Original magnification ×100 (A) or ×400 (B to H).

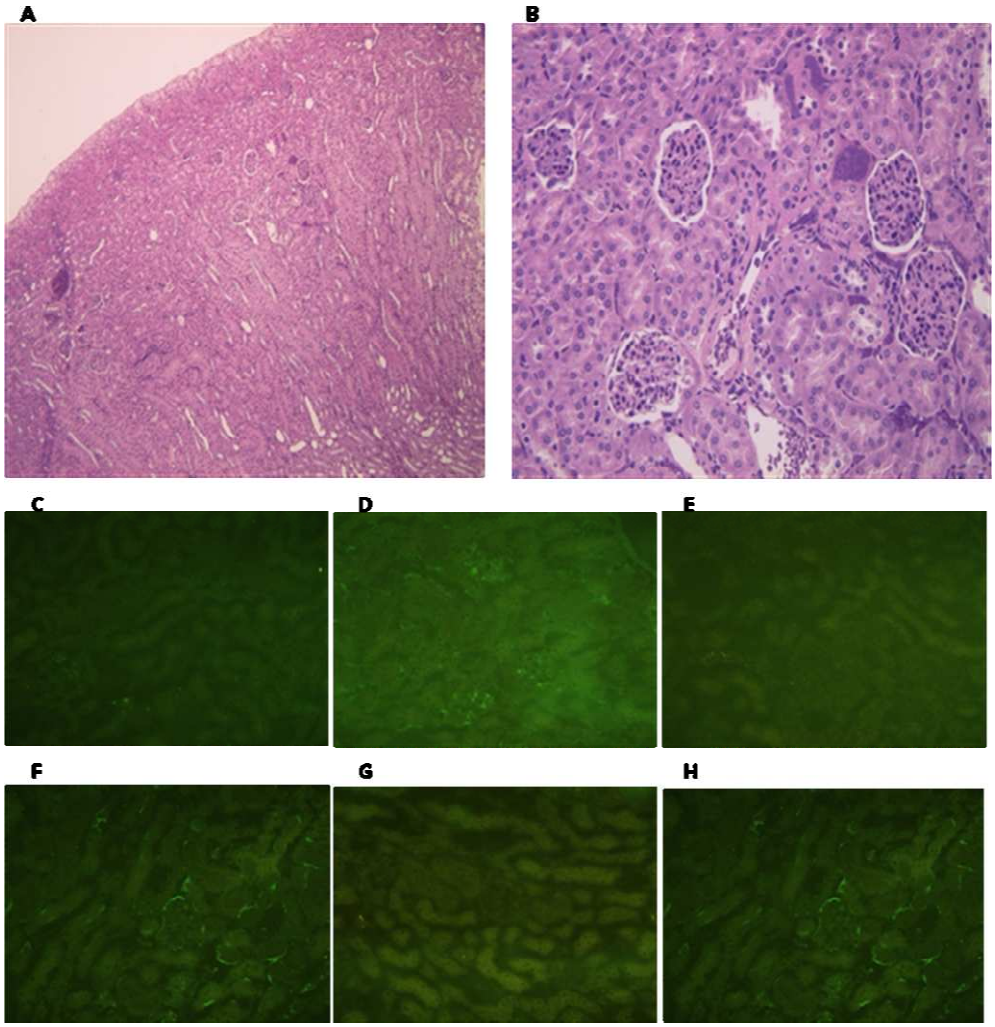


Figure 4. Renal histology of mouse CpG ODN (10 μg) group (16-week-old NZB/NZW F1 mouse) showing near normal findings. Cortical sections from representative mouse of mouse CpG ODN group were stained with H&E (A and B), IF for IgG (C), IgM (D), IgA (E), C1q (F) C3 (G), and fibrinogen (H). Original magnification ×100 (A) or ×400 (B to H).

4.1.5. Antibody test

4.1.5.1. Autoimmune antibodies: anti-dsDNA and anti-cardiolipin Abs

I traced the presence of anti-dsDNA and anti-cardiolipin antibodies (Abs) for autoimmunity test. To measure the antibodies, I obtained blood from retroorbital vein weekly before intraperitoneal injection and kept the sample sera in -70℃ deep freezer until antibody experiment. Figure 5A showed the time course of anti-dsDNA Ab production in NZB/NZW F1 mice received with mouse CpG ODN 10 μg for 4 weeks. Anti-dsDNA Ab level was augmented in mouse CpG ODN group only. In mouse CpG ODN group anti-dsDNA Ab was detected 50 dilutions of sera from the final sacrifice, and rose three-fold higher than control group (Fig. 5B). In parallel, anti-cardiolipin titer was measured in final blood sera taken after 5 weeks of each CpG ODN injection. Anti-cardiolipin Ab titer in mouse CpG ODN group rose eight-fold high compared to control group (Fig. 5C).

4.1.5.2. IgG_1 and IgG_{2a} antibodies

To verify switching of IgG subclass such as IgG_1 and IgG_{2a}, I assessed pre- and post-CpG ODN treatment sera of 5 week-aged NZB/NZW F1 mice by using ELISA. In mouse CpG ODN group, pre-post difference of IgG_1 was not big when compared to other group (Fig. 6A), whereas pre-post difference of IgG_{2a} titer appeared the biggest among all group (Fig. 6B). This switching IgG_{2a} might be due to Th1 skewed immune response following CpG ODN treatment.

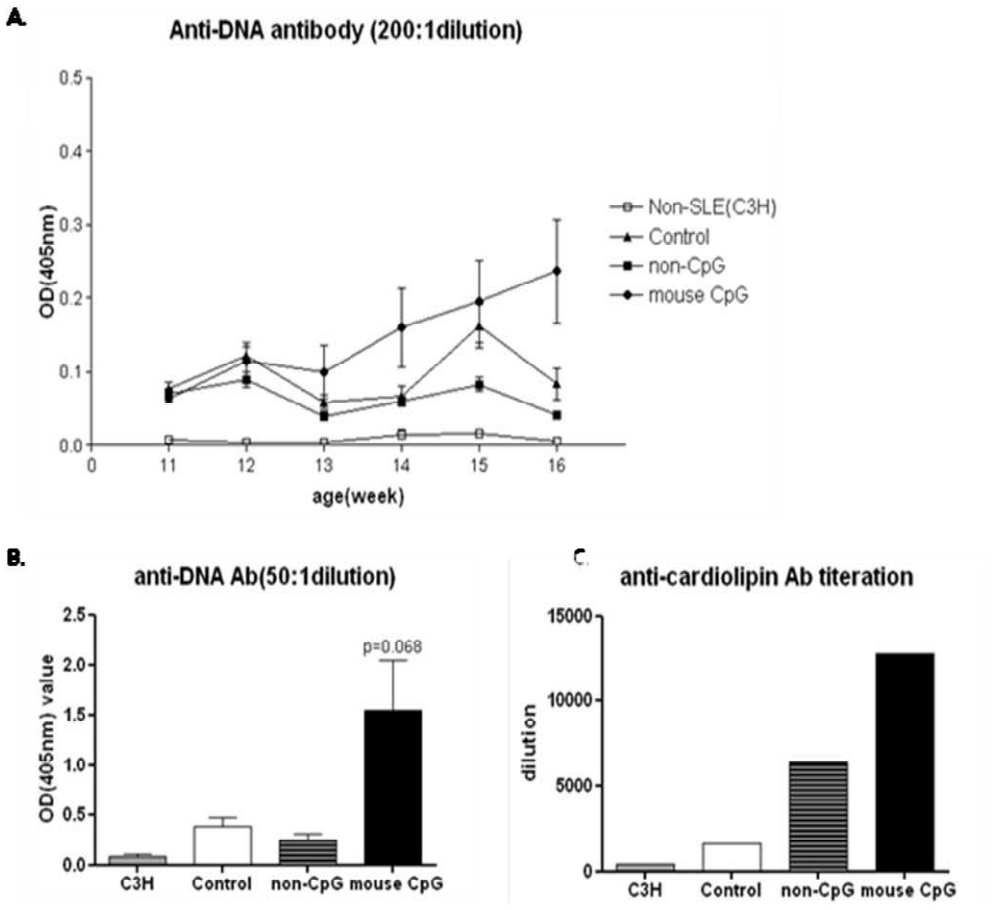


Figure 5. Anti-dsDNA (A, B) and anti-cardiolipin titers (C) autoantibody production in optimal dose of CpG ODN-treated NZB/NZW F1 mice. I used for negative control, autoantibody free C3H mice for same periods. At each time point, sera collected from the blood of each mouse were tested for the presence of IgG antibody agaisnt dsDNA by ELISA (A). Anti-cardiolipin titers was measured in final blood sera taken after 5 weeks of each CpGODN (C). Data were shown as mean±SD and each group is an individual measurement (n=21).

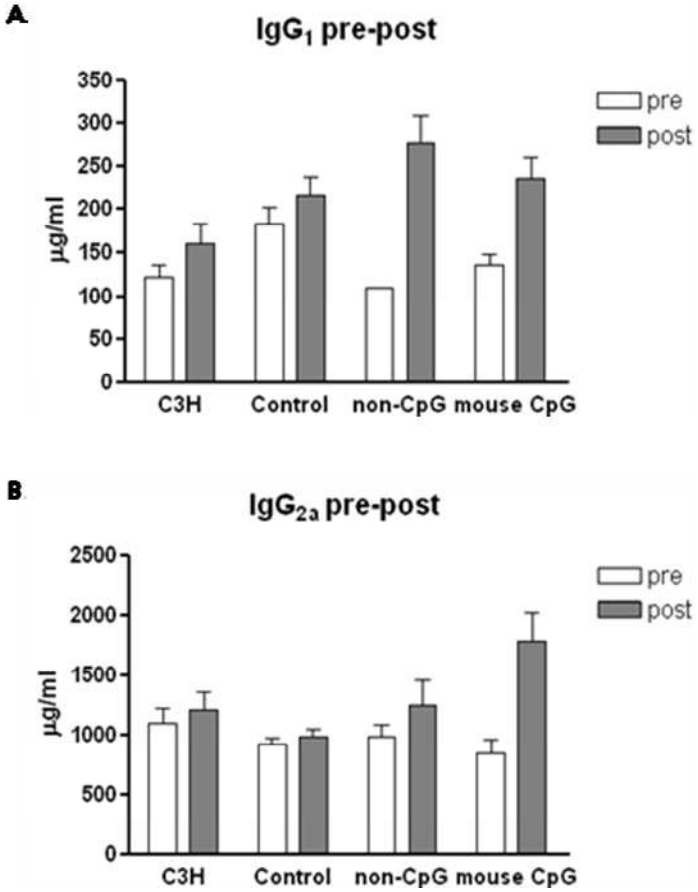


Figure 6. IgG_1 (A) and IgG_{2a} (B) production in optimal dose of CpG ODN-treated NZB/NZW F1 mice. I used for negative control, autoantibody free C3H mice for same periods. Before treated and after 4 weeks later sera were measured for the presence of IgG_1 and IgG_{2a} by ELISA. Data were shown as mean±SD and each group is an individual measurement (n=21).

4.1.6. Leukocyte subpopulations from spleen

To determine the leukocyte subpopulations from spleen such as B/T lymphocytes, NK cell, monocytes, and DC, flow cytometric analyses were adopted. Fig. 7A and 7B revealed that the proportion of B cells in mouse CpG-treated group was lower than that in control group. By contrast, the proportion of B cells in mouse CpG-treated group was higher than that in control group. As for the proportion of NK cells, monocytes, or granulocytes marker, CD11c+, there were little differences among all test groups (Fig. 7B). Meanwhile, the proportion of I-A/I-E (MHC class II marker) + cells in mouse or non-CpG ODN group was lower than that in control group (Fig. 7C). The proportion of costimulator B7-1 (makers for antigen presenting cell, dendritic cell, activated B cell and macrophage) + cells was lower in mouse CpG group than that in control group (Fig. 7D). Taken together, CpG ODN treatment renders B cell population in spleen of lupus-prone mice to decline, albeit T cell population to expand. These results were contradictory to the known effect (B cell proliferation and recruitment) of mouse CpG ODN.

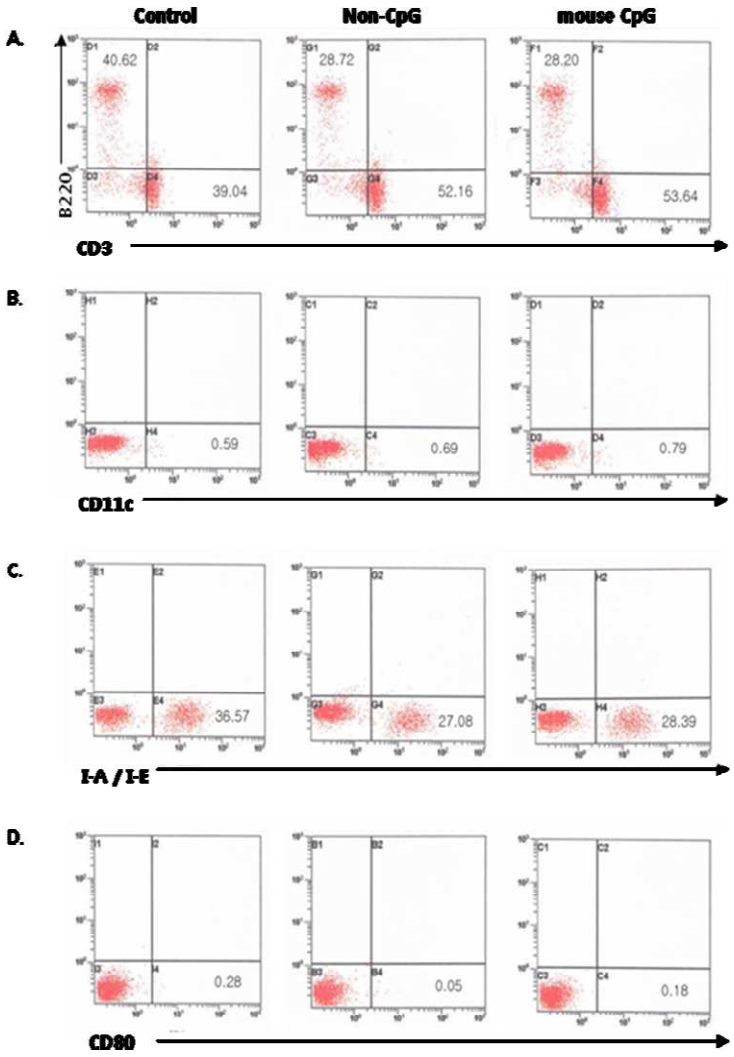


Figure 7. Flow cytometric analyses for CD45R/B220+ B and CD3+ T cell (A), CD11c+ cell (B), I-A/I-E+ cells (C), and CD80+ cells (D) on splenocytes in optimal dose of CpG ODN-treated NZB/NZW F1 mice. After sacrificed each mouse, Splenocytes were freshly isolated and stained with PE-anti-CD45R/B220 and FITC-anti-CD3, FITC-anti-CD11c, FITC-anti-I-A/I-E, or FITC-anti-CD80. Each data was shown as representative sample of individual measurement in each group.

4.1.7. Cytokine mRNA expressions in splenocytes

Previous study showed that as the course of SLE disease progress, the mRNA expression of IL-10, IFN-γ, TNF-α, IL-6 or IL-1 rises in splenocytes or serum albeit decline of IL-12 expression (129-131). Our RT-PCR data showed that in IFN-γ (Fig. 8A) and IL-10 (Fig. 8C) cytokine mRNA expressions in mouse CpG ODN group were slightly lower than in control mice group, whereas TNF-α (Fig. 8B) cytokine mRNA expression was slightly higher.

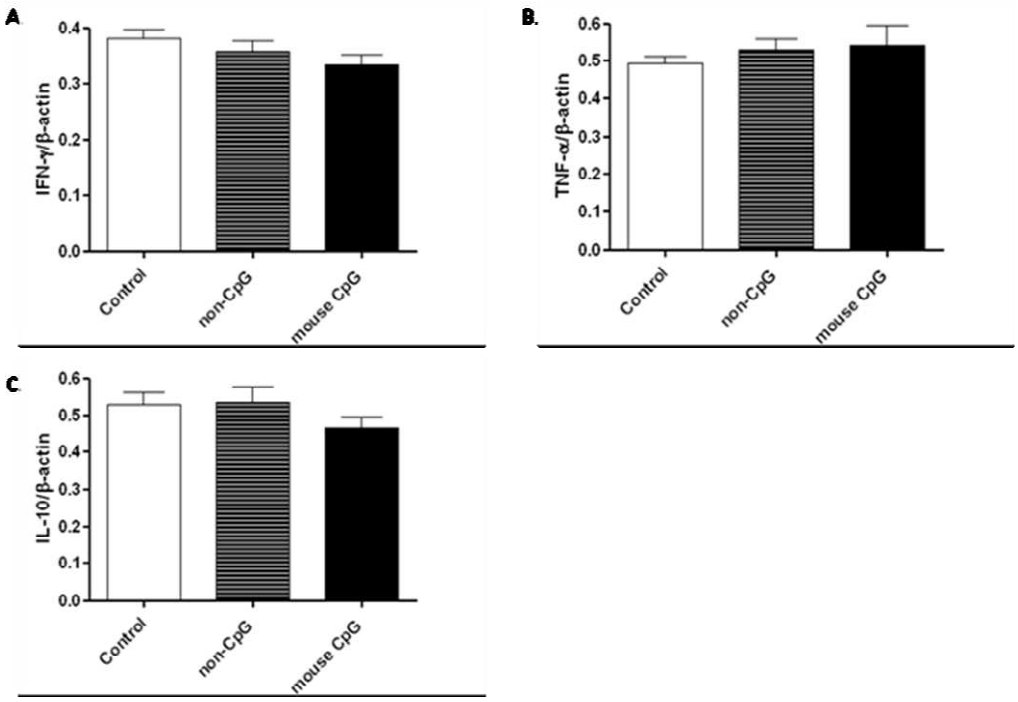


Figure 8. IFN-γ (A), TNF-α (B), and IL-10 (C) mRNA expressions on splenocytes in optimal dose of CpG ODN-treated NZB/NZW F1 mice. Cytokine mRNA expressions were determined by semiquantative RT-PCR. Data were shown as mean±SD and each group is an individual measurement (n=18).

4.2. The 2nd Experiment results: escalated dosing of CpG ODN treatment

First experiment to delineate the immunotoxicity of mouse CpG ODN in lupus-prone mice failed to prove the differentiality of immunotoxicity between control and mouse CpG ODN groups in terms of clinical and immune cell function parameters. However, the interesting point is that mouse CpG ODN-treated NZB/NZW F1 group displayed less severe or more attenuating index when gauging immune cell and cytokines parameter as a major index of serious SLE disease. To reconfirm mouse CpG ODN-induced immunotoxicity, I initiated dose- escalating experiment, where mice were weekly i.p. treated with escalated dose of mouse CpG ODN for 8 week, and observed for 12 week to compare starting point of proteinuria without any intervention.

4.2.1. BW and Organ weight ratios

To determine the immunotoxicological effects in NZB/NZW F1, following CpG ODN injection, I measured BW and organ weight/BW ratios of female lupus mice treated with mouse CpG ODN in escalated dose (10, 30, and 50 μg) or vehicle only for control group. BW (Fig. 9), liver/BW ratio (Fig. 10C) or lung/BW ratio (Fig. 10D) was not significantly different in groups even if all mouse CpG ODN-treated groups seemed more weighted. Interestingly, spleen/BW ratio (Fig. 10A) or kidney/BW ratio (Fig. 10B) was significantly different between control and mouse CpG ODN 30 μg or 50 μg ($p<0.05$). Spleen/BW ratio in mouse CpG ODN-treated group was higher than in control group. By contrast, kidney/BW ratio in mouse CpG ODN group was lower than in control group.

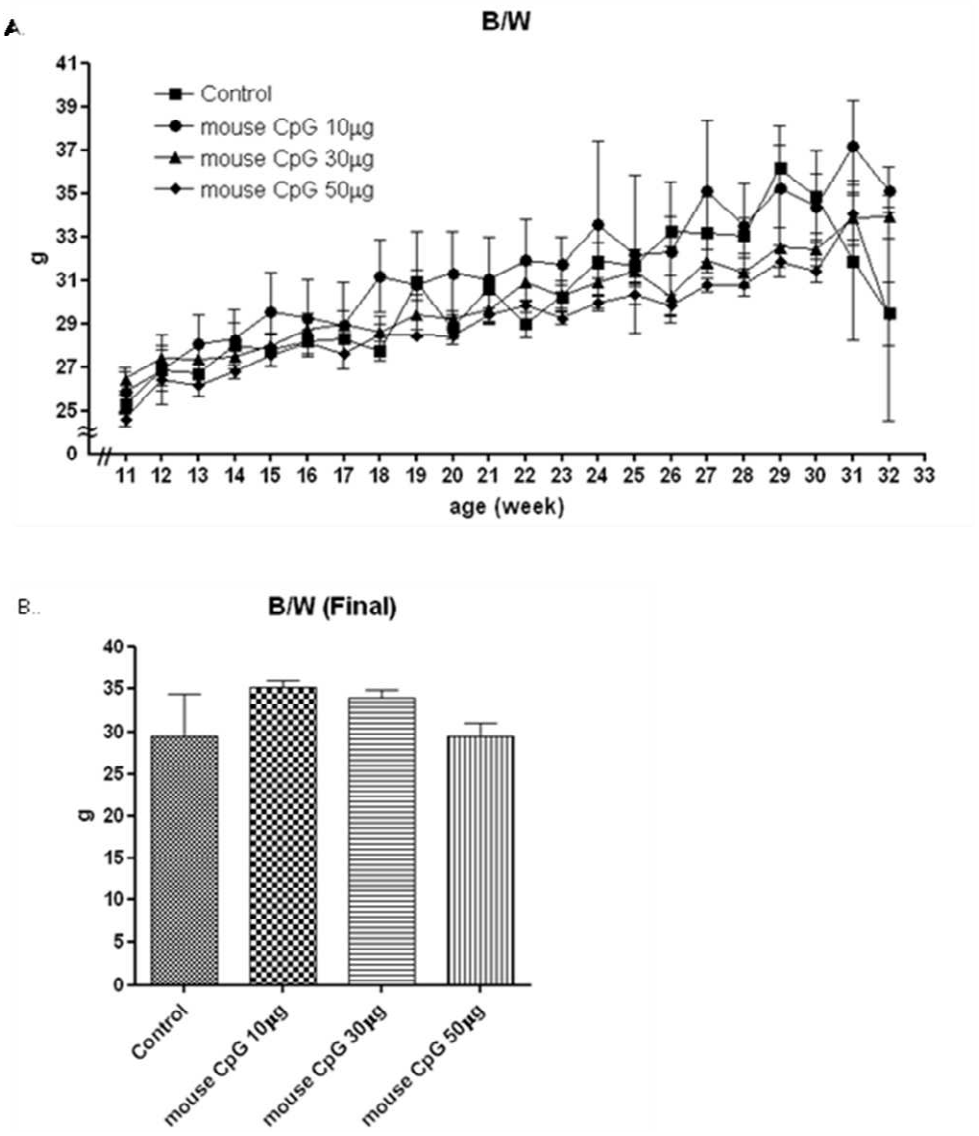


Figure 9. . BW change in escalated dose of CpG ODN treated-NZB/NZW F1 mice. Data were shown as mean±SD and each group is an individual measurement (n=18).

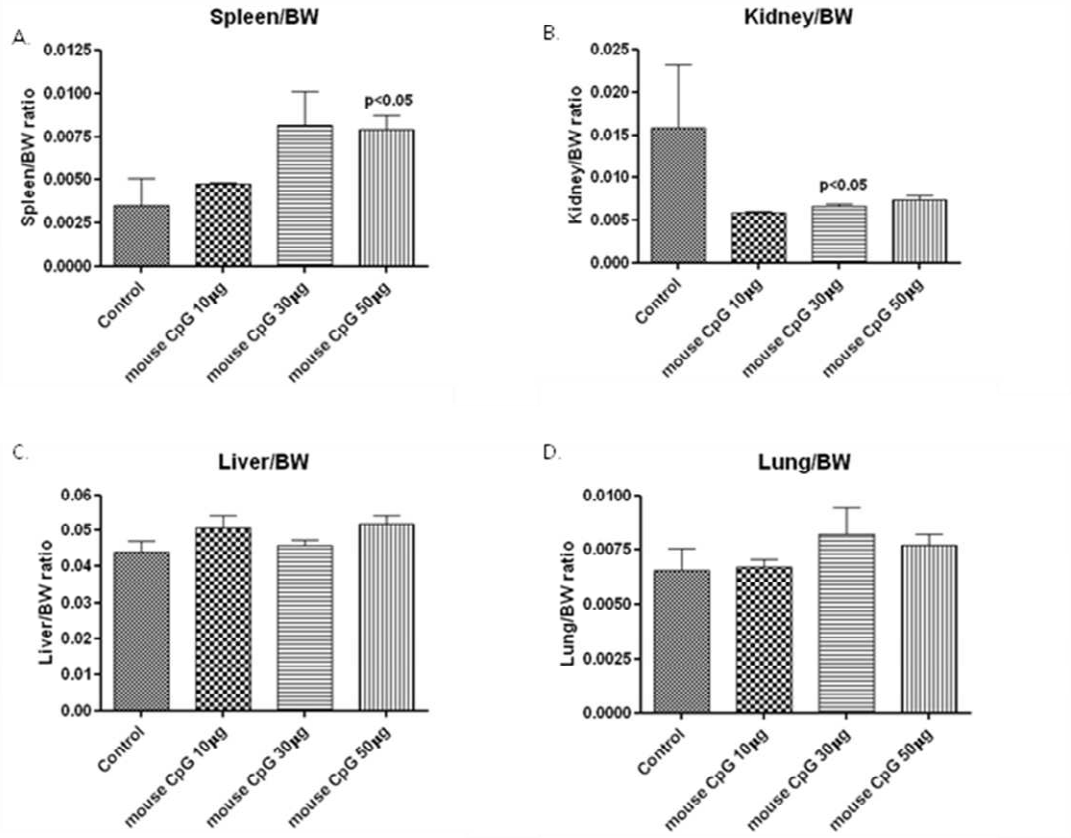


Figure 10. Organ/BW ratios in escalated dose of CpG ODN treated-NZB/NZW F1 mice. After sacrifice each mouse were measured for weight of spleen, kidney, liver, and lung organ. Data were shown as mean±SD and each group is an individual measurement (n=18).

4.2.2. Hematological parameters

To investigate hematopoietic impact of CpG ODN in lupus-prone mouse model, I performed WBC with differential counts, erythrocyte counts, and platelet counts for hematology function. For serum biochemical analyses, I measured BUN, Cr, BUN/Cr ratio for kidney function.

Among groups, the counts of WBC, neutrophil (NE), lymphocytes (LY), monocytes (MO), eosinophil (EO), basophil (BA) in mouse CpG ODN (30μg) group were two-fold or higher, whereas the percentage of leukocytes were within normal range (Table 14). There were no significant changes in leukocyte count among all groups. Contrary to the first experiment result of erythrocytes in Table 15, CpG-untreated control group showed overt anemia by lower hemoglobin, hematocrit, and MCV levels. In addition, control group manifested only thrombocytopenia in blood counts (Table 16).

To investigate renal impact of CpG ODN in lupus-prone mouse model, I measured BUN, Cr, and BUN/Cr ratio for renal function. Contrary to the first experiment, increased BUN of all groups showed damaged renal function but there were no significant differences among all groups (Table 17)

Table 14. Leukocyte counts in escalated dose of CpG ODN treated-NZB/NZW F1 mice

	Normal range	Disease free SLE mice	Control (n=3)	Mouse CpG 10 μg (n=3)	Mouse CpG 30 μg (n=6)	Mouse CpG 50 μg (n=6)	X^2	p
		MEAN(SD)						
WBC (K/$\mu\ell$)	1.8~10.7	5.84(0.741)	4.11(3.007)	4.02(1.500)	10.53(5.193)	4.47(2.206)	6.930	0.074
NE (K/$\mu\ell$)	0.1~2.4	1.38(0.322)	1.69(1.653)	0.98(0.501)	3.06(1.710)	1.18(0.526)	6.460	0.091
LY (K/$\mu\ell$)	0.9~9.3	3.94(0.425)	2.25(1.556)	2.75(1.141)	6.69(2.968)	3.10(1.748)	6.632	0.085
MO (K/$\mu\ell$)	0.0~0.4	0.32(0.197)	0.13(0.040)	0.23(0.156)	0.43(0.223)	0.17(0.058)	5.693	0.128
EO (K/$\mu\ell$)	0.0~0.2	0.15(0.119)	0.03(0.000)	0.04(0.036)	0.28(0.306)	0.02(0.008)	3.109	0.375
BA (K/$\mu\ell$)	0.0~0.2	0.05(0.033)	0.00(0.006)	0.01(0.015)	0.07(0.096)	0.01(0.005)	2.509	0.474
NE (%)	6.6~38.9	23.45(3.338)	36.46(13.455)	25.65(10.813)	28.44(5.117)	27.22(6.899)	0.895	0.827
LY (%)	55.8~91.6	67.78(6.228)	56.53(11.072)	67.77(7.948)	65.04(6.789)	68.09(7.227)	3.380	0.337
MO (%)	0.0~7.5	5.37(3.075)	5.53(4.924)	5.30(2.727)	4.11(1.353)	4.11(1.635)	0.433	0.933
EO (%)	0.0~3.9	2.57(1.864)	1.43(1.550)	0.93(0.653)	1.93(1.847)	0.43(0.311)	1.739	0.628
BA (%)	0.0~2.0	0.84(0.520)	0.05(0.057)	0.35(0.302)	0.47(0.564)	0.16(0.102)	3.354	0.340

Table 15. Erythrocyte counts in escalated dose of CpG ODN treated-NZB/NZW F1 mice

	Normal range	Disease free SLE mice	Control (n=3)	Mouse CpG 10 μg (n=3)	Mouse CpG 30 μg (n=6)	Mouse CpG 50 μg (n=6)	X^2	p
		MEAN(SD)						
RBC (M/$\mu\ell$)	6.36~9.42	8.96(1.464)	7.17(3.075)	9.43(0.752)	8.54(2.136)	8.37(2.178)	1.392	0.707
Hb (g/dℓ)	11.0~15.1	12.12(1.839)	9.43(4.461)	12.70(2.265)	12.27(2.368)	11.63(3.132)	1.860	0.602
HCT (%)	35.1~45.4	48.22(7.787)	34.33(15.426)	48.63(6.274)	45.87(9.479)	43.83(12.468)	2.229	0.526
MCV (fL)	45.4~60.3	53.82(1.393)	47.67(1.242)	51.47(2.818)	54.57(8.422)	52.03(3.357)	5.355	0.148
MCH (pg)	14.1~19.3	13.78(3.099)	12.97(0.737)	13.40(1.562)	14.60(1.786)	13.88(0.627)	2.983	0.394
MCHC (g/dℓ)	30.2~34.2	25.53(5.161)	27.23(0.808)	26.00(1.652)	26.88(1.179)	26.70(0.713)	1.056	0.788
RDW (%)	12.4~27.0	18.07(0.753)	20.70(1.044)	20.27(0.666)	20.43(1.256)	20.78(3.176)	1.842	0.606

Table 16. Platelet counts in escalated dose of CpG ODN treated-NZB/NZW F1 mice

	Normal range	Disease free SLE mice	Control (n=3)	Mouse CpG 10 μg (n=3)	Mouse CpG 30 μg (n=6)	Mouse CpG 50 μg (n=6)	X^2	p
		MEAN(SD)						
PLT (K/$\mu\ell$)	592~2972	650.83(166.150)	356.67(399.903)	860.67(204.962)	643.00(278.540)	646.00(149.164)	4.105	0.250
MPV (fL)	5.0~20.0	4.97(0.280)	6.23(1.001)	5.60(0.361)	6.17(0.497)	6.12(0.454)	2.889	0.409

Table 17. Serum renal function tests in escalated dose of CpG ODN treated-NZB/NZW F1 mice

	Normal range	Disease free SLE mice	Control (n=3)	Mouse CpG 10 μg (n=3)	Mouse CpG 30 μg (n=6)	Mouse CpG 50 μg (n=6)	X^2	p
		MEAN(SD)						
BUN (mg/dl)	9.2~29.2	18.68(3.191)	178.17(168.949)	57.30(60.640)	35.90(28.758)	69.12(98.804)	3.965	0.265
Cr (mg/dl)	0.4~1.4	0.30(0.000)	0.67(0.451)	0.23(0.153)	0.27(0.163)	0.27(0.137)	2.788	0.426
BUN/Cr ratio	6.57~73	62.28(10.636)	231.29(87.341)	215.08(99.814)	152.99(74.521)	207.78(169.100)	1.959	0.581

4.2.3. Proteinuria

To define the degree of SLE progression in NZB/NZW F1 mice, I checked urinary protein level as sensitive index (Fig. 11). As urinary protein level is above 3.33 mg/ml, it refers to serious renal damage. First, in mouse CpG (10 μg) group, proteinuria started on 23 week of age, and soon on next week, the mouse had a dyspnea and rapid weight-gain. After the mouse euthanized on 24 week of age, I inspected the mouse abdomen, and knew that primary cause of dyspnea was huge amount of abdominal fluid. After 24 week of age, mouse CpG ODN (10 μg) group did not reveal any more proteinuria until the end of this experiment. Next, in control group proteinuria began to appear on 27th and on 30th week. Control group mice showed same dyspnea and weight-gain, but symptom was less severe than the first mouse showing proteinuria.

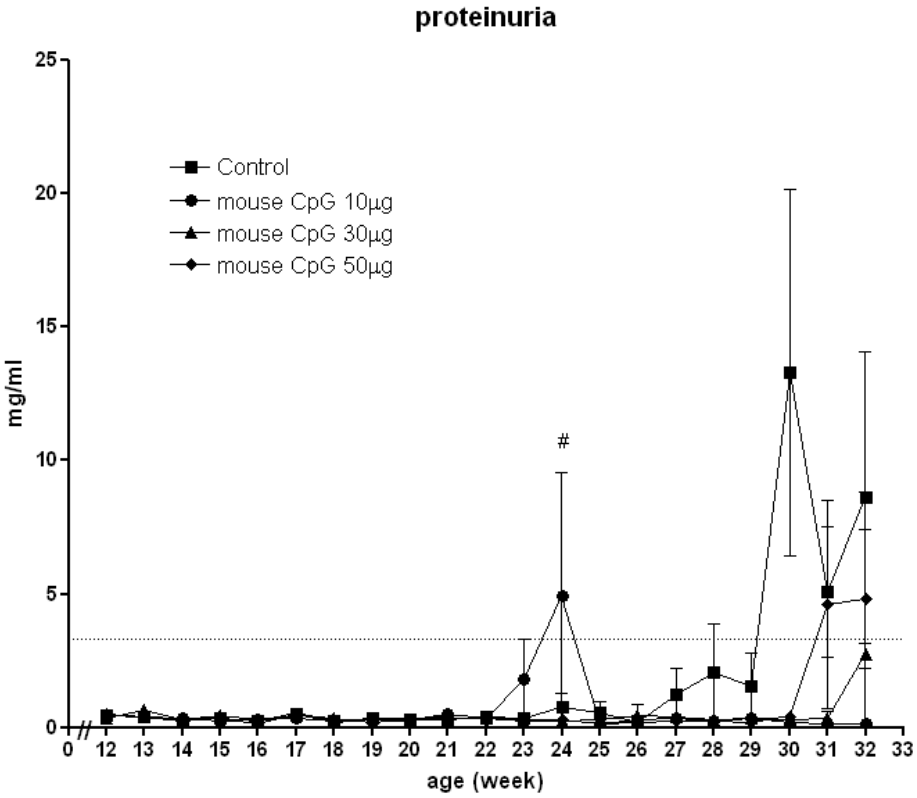


Figure 11. Proteinuria test in escalated dose of CpG ODN treated-NZB/NZW F1 mice. At each time point, spot urine collected from each mouse was tested for the presence of protein in urine by Bradford assay. Data were shown as mean±SD and each group is an individual measurement (n=18). Above 3.3 mg/ml of urinary protein level refers to severe nephritis.

#: a mouse of CpGODN (10 μg) group euthanized on 24 week of age because of dyspnea with large amount of abdominal fluid.

4.2.4. Renal histopathology

To verify glomerularnephritis following CpG ODN injection, I examined H&E, immunofluorescence (IF) microscopy and electron microscopy (EM) at the 32nd week.

In control group, the golmeruli showed multifocal crescent formation and diffuse hypercellularity due to to mild mesengial cell proliferation. There were a few lymphocytic infiltrations in interstitium and the tubles and blood vessels were unremarkable (Fig. 12A). On the immunofluorescent findings, mesangia revealed weak positive deposits of IgG (Fig. 12B), IgM, and C3 (Fig.12C; Table 18). On the electron microscopic findings, glomerular basement membranes (GBMs) were normal thickness and mesangia were irregularly widened by few electro-dense deposits (EDDs) (Fig. 12D).

In mouse CpG (10 μg) group, the glomeruli were diffusely enlarged and capillary lumens were narrowed due to mesangial and endothelial cell proliferation. The tubule, blood vessel, and interstitium were unremarkable (Fig. 13A). The immunofluorescent findings revealed weak IgG (Fig. 13B), IgM, C3 (Fig. 13C) deposits in the mesania (Table 18). On the ultrastructural findings, GBMs were normal thickness and the mesangia were irregularly widened by numerous EDDs of variable sizes (Fig. 13D).

In mouse CpG (30 μg) group, there were diffuse glomerular enlargement due to mesangial cell proliferation and luminal narrowing of capillary loops. Interstitium showed a few lymphocytic infiltrations. The tuble and blood vessel were unremarkable (Fig. 14A). On the immunofluorescent findings, weak mesangial IgG (Fig. 14B), IgM, and C3 (Fig. 14C) deposits were noted (Table 18). On the electron microscopy, GBMs were normal thickenss and the mesangia were markedly widened by many EDDs of variable sizes with paramesangial

extension. The epithelia revealed multifocal foot process fusion and villous transformation (Fig 14D).

In mouse CpG (50 μg) group, the glomeruli were diffusely enlarged with mutifocal crescent formation or periglomerular fibrosis. There were a few lymphocytic infiltration and the tubule and blood vessel were unremarkable (Fig. 15A). On the immunofluorescent findings, the mesangia revelaed weak intensity of IgG (Fig 15B), IgM, C3 (Fig. 15C), and fibrinogen deposits (Table 18). On the ultrastructural findings, glomerular basement membranes (GBMs) were normal thickness and the mesangia were slightly widened by afew electron-dense deposits (EDDs). The epithelia revealed diffuse effacement of foot processes (Fig. 15D).

Through all groups, I could classify as diffuse proliferative glomerulonephritis (Class IV), according to WHO classification of lupus nephritis although the severity seemed to be increased according to the increase of dose of CpG ODN.

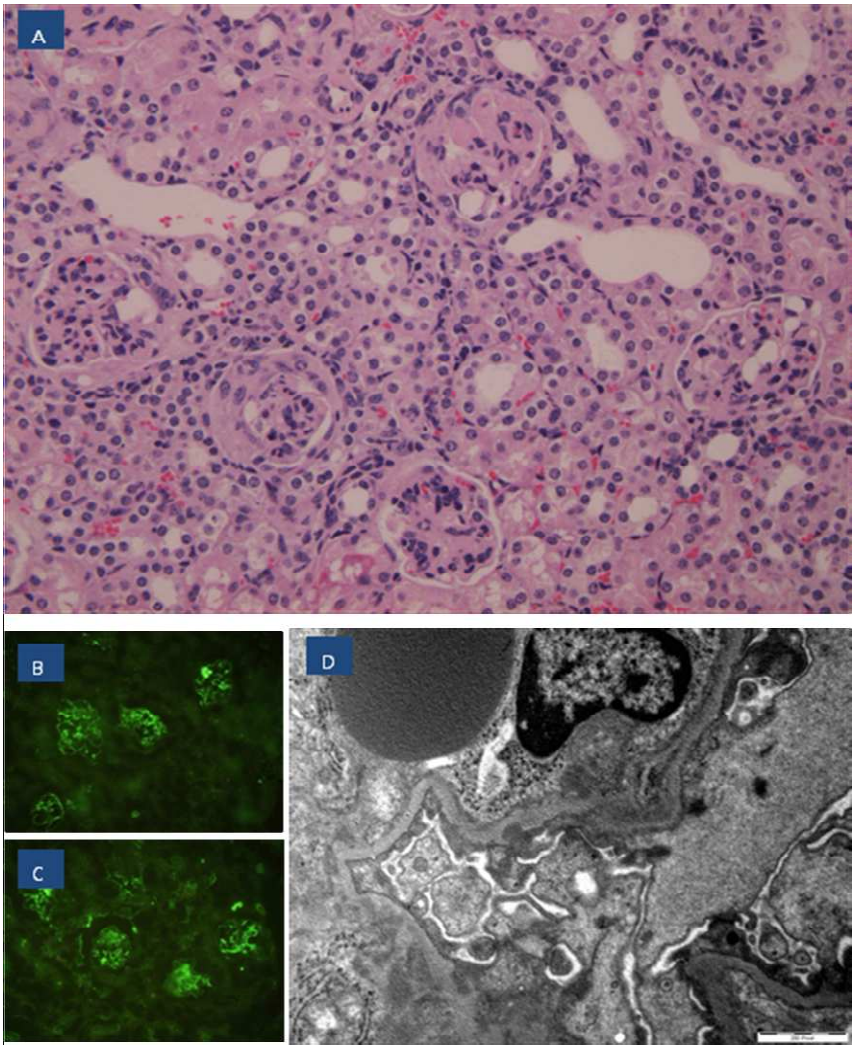


Figure 12. Renal histopathology of control group in 32-weeks-old NZB/NZW F1 mouse showing diffuse proliferative glomerulonephritis with crescent, weak mesangial IgG & C3 deposits and few mesangial electron dense deposits. Cortical sections from the representative mouse of control group were stained with H&E (A), IF for IgG (B) and C3 (C), and EM (D). Original magnification ×400 (A to C) or ×5000 (D).

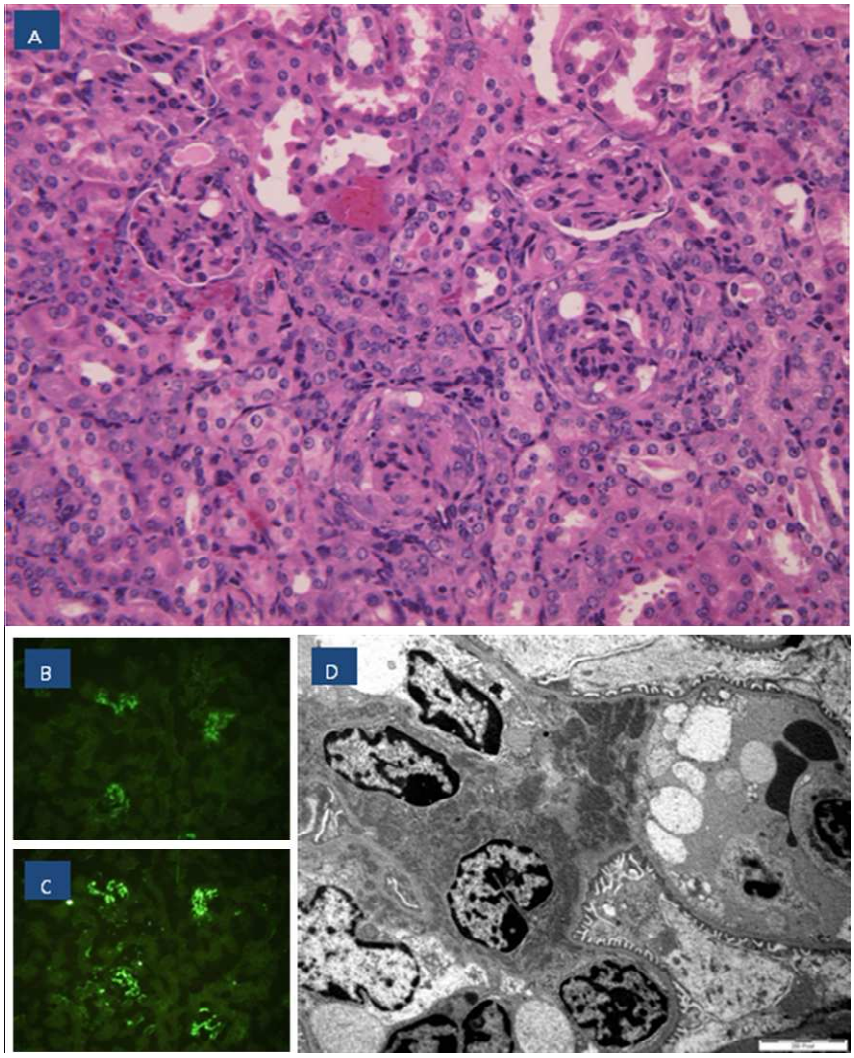


Figure 13. Renal histopathology of mouse CpG ODN (10 μg) group in 32-weeks-old NZB/NZW F1 mouse showing diffuse proliferative glomerulonephritis with crescents, moderate mesangial IgG & C3 deposits and numerous mesangial electron dense deposits. Cortical sections from the representative mouse of control group were stained with H&E (A), IF for IgG (B) and C3 (C), and EM (D). Original magnification ×400 (A to C) or ×5000 (D).

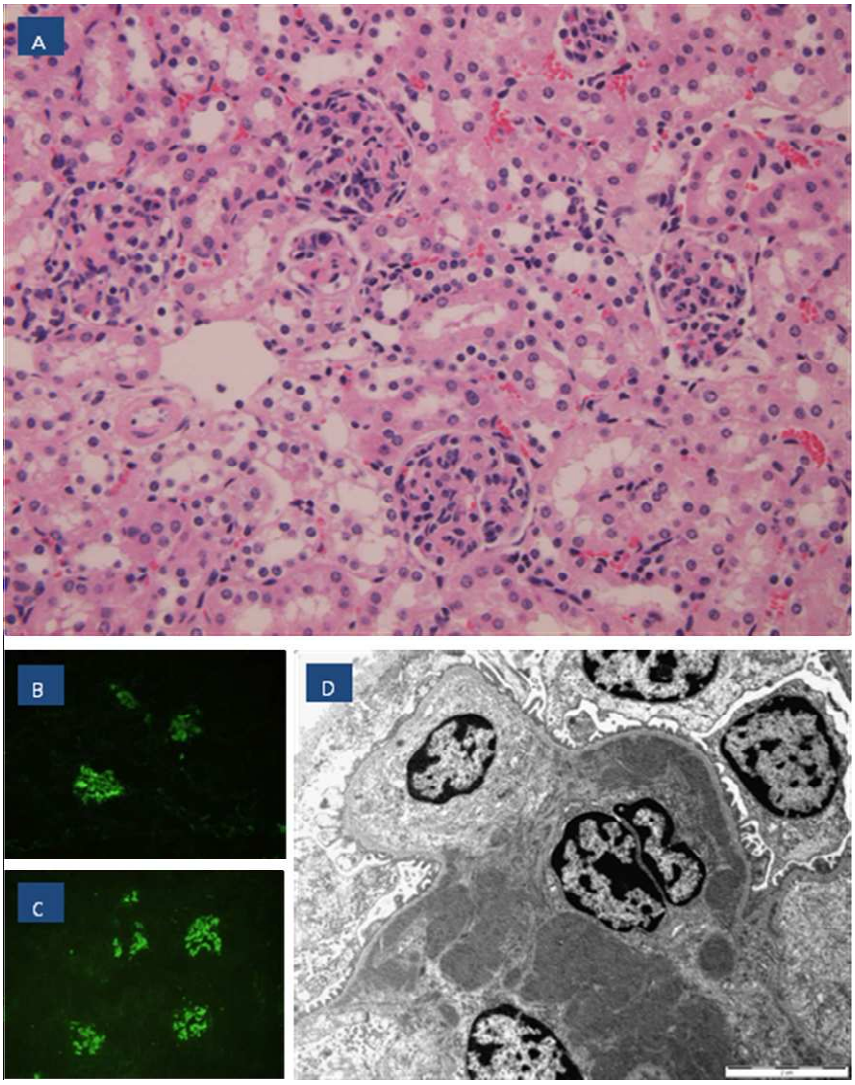


Figure 14. Renal histopathology of mouse CpG ODN (30μg) group in 32-weeks-old NZB/NZW F1 mouse showing diffuse proliferative glomerulonephritis with crescents, moderate mesangial IgG & C3 deposits and numerous mesangial electron dense deposits. Cortical sections from the representative mouse of control group were stained with H&E (A), IF for IgG (B) and C3 (C), and EM (D). Original magnification ×400 (A to C) or ×5000 (D).

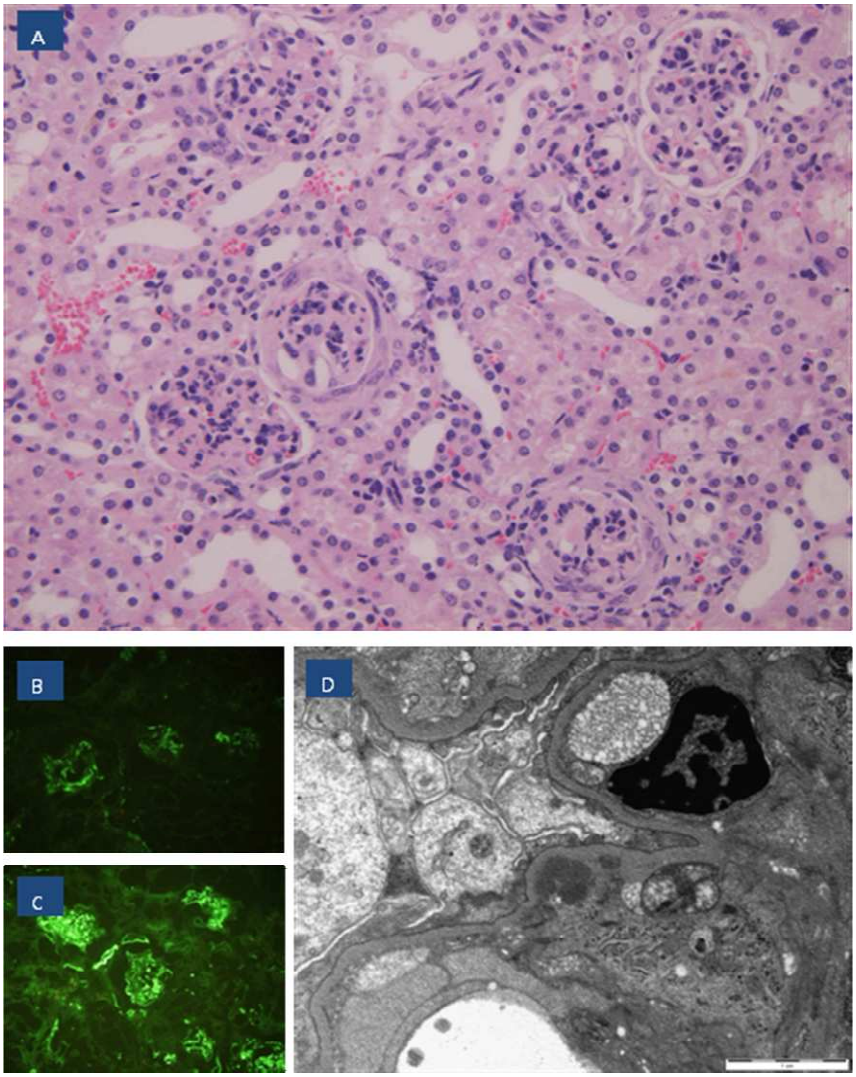


Figure 15. Renal histopathology of mouse CpG ODN (50 μg) group in 32-weeks-old NZB/NZW F1 mouse showing diffuse proliferative glomerulonephritis with crescents, moderate mesangial IgG & C3 deposits and mesangial electron dense deposits. Cortical sections from the representative mouse of control group were stained with H&E (A), IF for IgG (B) and C3 (C), and EM (D). Original magnification ×400 (A to C) or ×5000 (D).

Table 18. Immunofluorescence detection in mesangium of kidney*

Antibodies	Control	Mouse CpG 10 μg	Mouse CpG 30 μg	Mouse CpG 50 μg
IgG	+	+	+	+
IgA	-	-	-	-
IgM	-	+	-	+
C3	+	+	+	+
C1q	-	-	-	-
Fibrinogen	-	-	-	+

- Negative; + Weak positive; ++ Moderate positive; +++ Strong positive

* Reading results of each representative mouse in Fig. 12~15

4.2.5. Autoimmune antibodies: anti-dsDNA and anti-cardiolipin DNA Abs

After I injected weekly eascalated dose of mouse CpG ODN (10, 30, and 50 μg) for 8 weeks, I observed without any treatment for 12weeks. To measure the these antibodies titers, I obtained blood from retroorbital vein weekly before intraperitoneal injection and kept the sample sera in -70 deep freezer until antibody experiment. Anti-dsDNA and –cardiolipin Abs were diluted 200 times from sera before measuring OD value in 405 nm. Fig. 16A and B indicated that titer of anti-dsDNA Abs in mouse CpG ODN group rose dose dependently Using sera from the final sacrifice of each group, titer of anti-dsDNA Ab in mouse CpG (10 μg) group was the highest among groups (Fig. 16C). Anti-cardiolipin Ab titer revealed CpG-dose dependence (Fig. 16D).

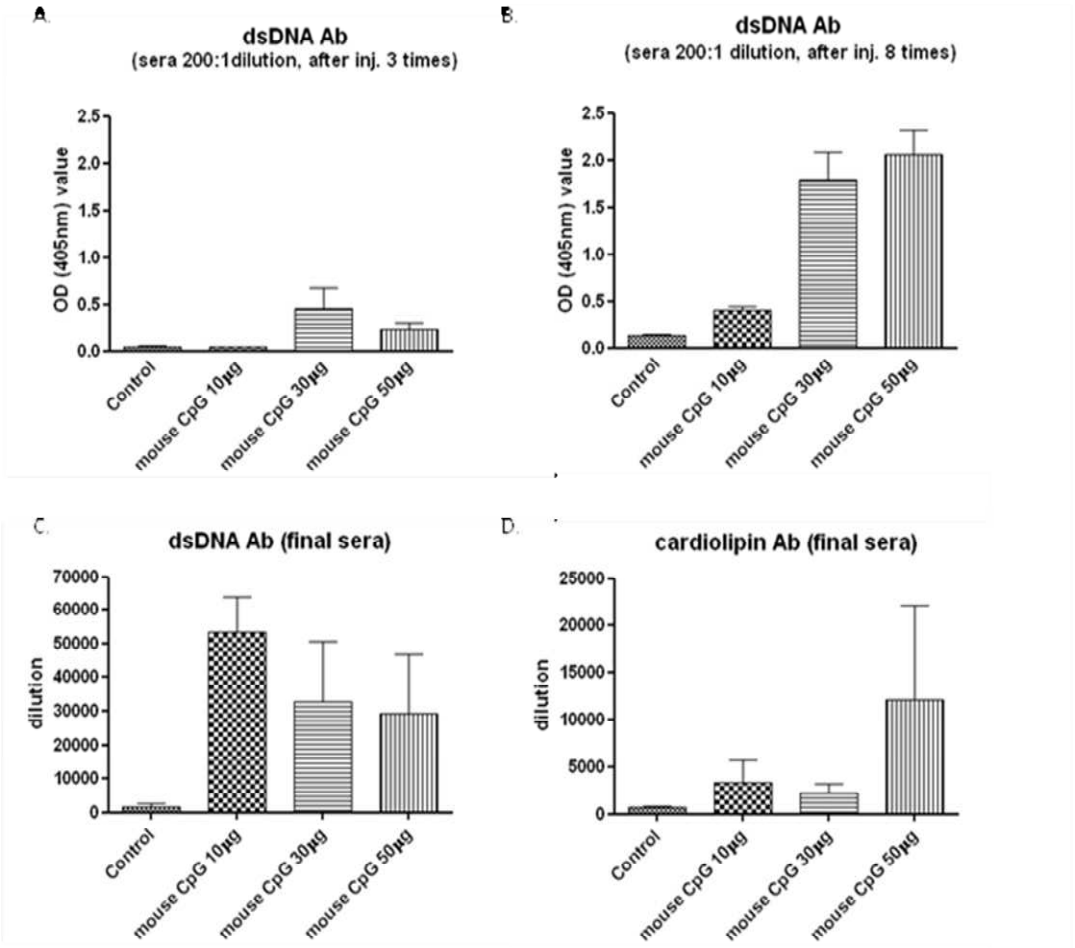
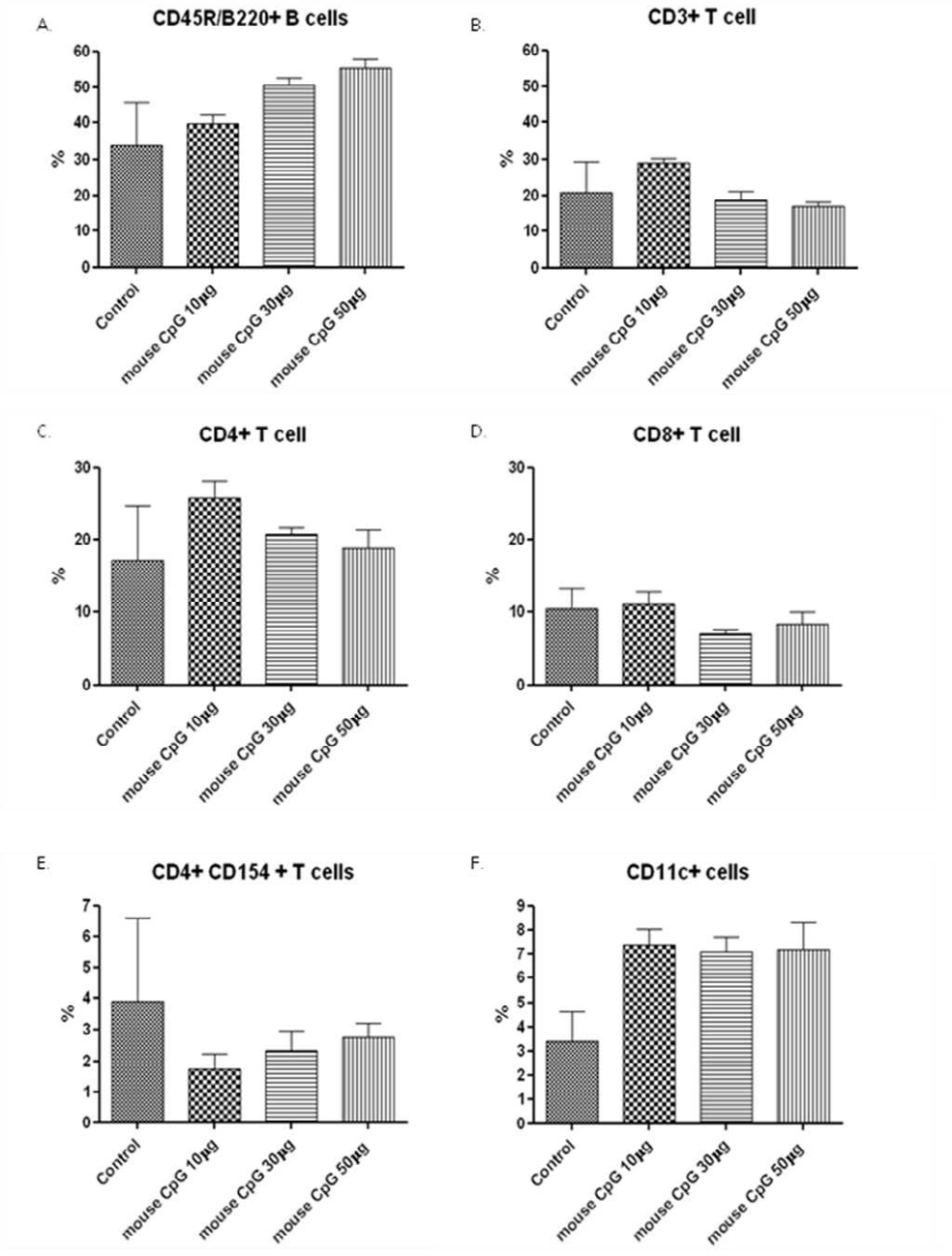


Figure 16. Anti-dsDNA (A, B, C) and anti-cardiolipin (D) autoantibody formation in escalated dose of CpG ODN treated-NZB/NZW F1 mice. At each time point, sera collected from the blood of each mouse were tested for the presence of IgG Ab against dsDNA or cardiolipin by ELISA. Data were shown as mean±SD and each group is an individual measurement (n=18).

4.2.6. Leukocyte subpopulations from spleen

Leukocyte subpopulations from spleen were detected by using flow cytometry. Contrary to the first experiment, the proportion of B (CD45R/B220+) cell in mouse CpG ODN group rose dose dependently (Fig. 17A). By contrast, the proportion of T cell, CD3+ T, CD8+ T and CD4+T cell appeared declined dose dependently (Fig. 17B, 17C, and 17D). As for the proportion of NK cells, monocytes, or granulocytes marker, CD11c+, there were two-fold or higher increase in all CpG groups than in control group (Fig. 17F). The proportion of I-A/I-E (MHC class II marker) + cells in mouse CpG group rose in dose dependent manner (Fig. 17I). Interestingly, the proportion of activated CD4 T cell (CD154+) in mouse CpG group was lower than in control group (Fig. 17E). The proportion of costimulator B7-1 (CD80+) and B7-2 (CD86+) positive/expressed cells were higher than in control group (Fig. 17G, 17H, and 17J).


A.
CD45R/B220+ B cells
%
Control
mouse CpG 10μg
mouse CpG 30μg
mouse CpG 50μg
B.
CD3+ T cell
C.
CD4+ T cell
D.
CD8+ T cell
E.
CD4+ CD154 + T cells
F.
CD11c+ cells

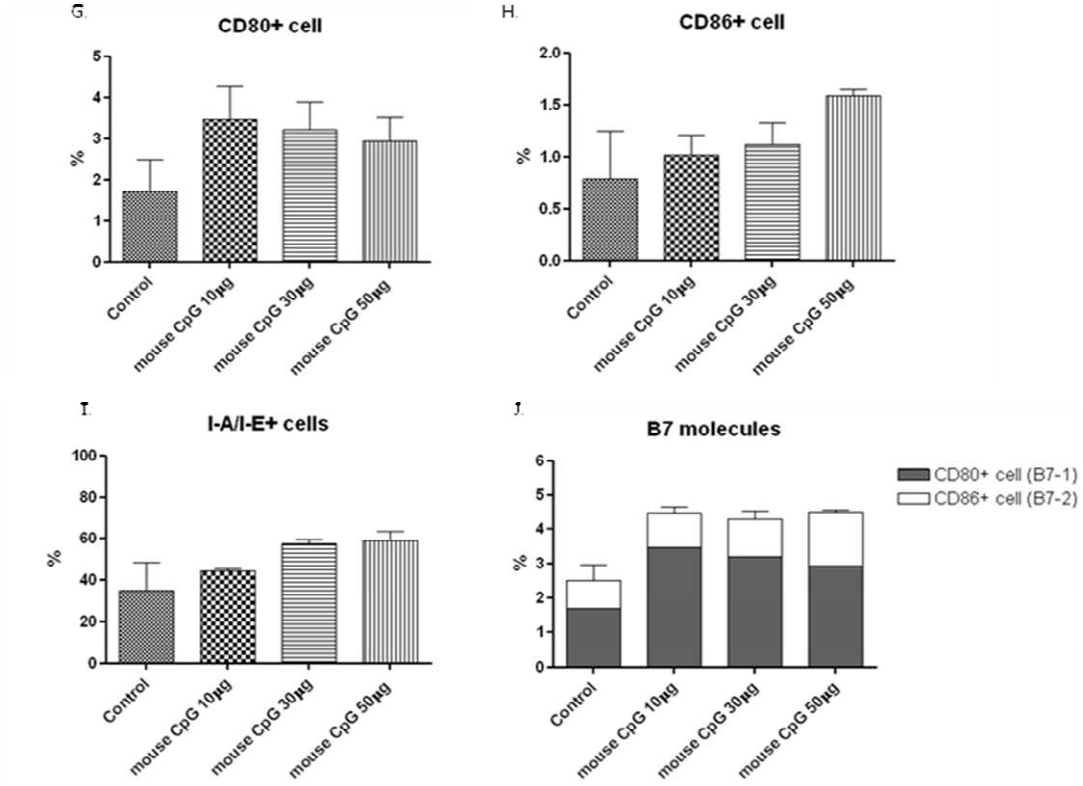


Figure 17. Flow cytometric analyses for CD45R/B220+ B cells (A), CD3+ T cells (B), CD4+ T cells (C), CD8+ T cells (D), CD154+CD4+ T cells (E), CD11c+ cells (F), CD80+ cells (G), CD86+ cells (H), I-A/I-E+ cells (I), and B7 molecules (J) on splenocytes in escalated dose of CpG ODN treated-NZB/NZW F1 mice. After sacrifice of mice, I freshly isolated splenocytes and stained with PE-anti-CD45R/B220 and FITC-anti-CD3, PE-anti CD8 and FITC-anti-CD4, PE-anti-CD154 and FITC-anti-CD4, FITC-anti-CD11c, FITC-anti-CD80, FITC-anti-CD86, or FITC-anti-I-A/I-E. Data were shown as mean±SD and each group is an individual measurement (n=18).

4.2.7. Cytokine mRNA expressions in splenocytes

I examined IFN-γ (Fig. 18A), IL-2 (Fig. 18B), TNF-α (Fig.18C), and IL-10 (Fig. 18D) mRNA expressions in splenocytes of lupus-prone NZB/NZW F1 mice challenge with escalated dose of mouse CpG. Our RT-PCR data showed that in IFN-γ cytokine mRNA expression in mouse CpG group (50μg) was significantly lower than control mice group. In general, all tested cytokine (IFN-γ, IL-2, TNF-α, and IL-10) mRNA expressions in CpG group appeared higher than control mice group.

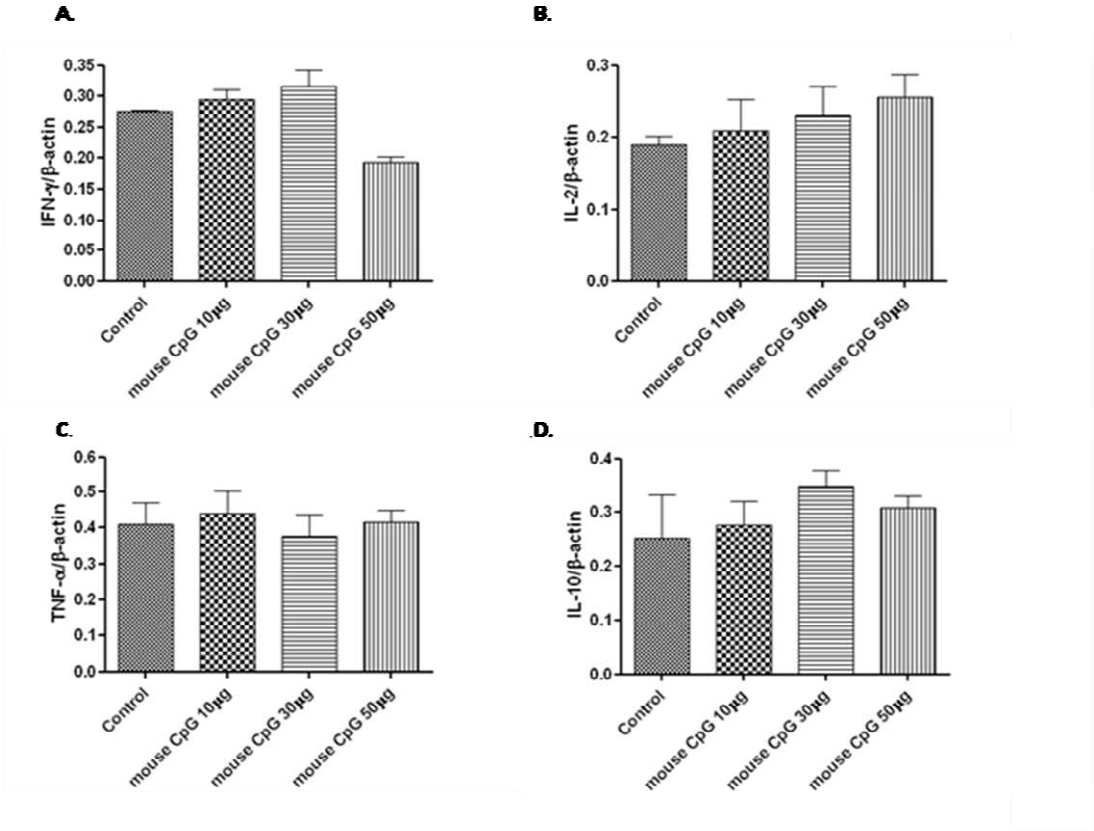


Figure 18. IFN-γ (A), IL-2 (B), TNF-α (C), IL-10 (D) mRNA expressions on splenocytes in escalated dose of CpG ODN treated-NZB/NZW F1 mice. Cytokine mRNA expressions were determined by semiquantative RT-PCR of total freshly isolated splenocytes. Data were shown as mean±SD and each group is an individual measurement (n=18).

4.2.8. Correlation between parameters of the second experiment

As SLE disease shows a wide array of clinico-immunopathological manifestations during active disease, it is worth to explore correlation among different parameters from the second escalated dosing experiment, possibly permitting to understand the progress of SLE disease. All of parameters individually measured results of each mouse in all groups.

By organ/BW ratio, spleen and kidney appeared relatively an increase in volume. To elucidate the contributing factor of enlarged volume of organ, I performed the correlation between spleen/BW ratio and each subpopulation lymphocyte in spleen, B cells (r=0.620, p=0.008), CD11c+ cells (r=0.651, p=0.005), B7-2 cells (r=0.752, p=0.000), and MHC class II, I-A/I-E+ cells (r=0.713, p=0.001), thus contributing to increased significant spleen/BW ratio, whereas total T (CD3+) cells (r=−0.466, p=0.060) might be decreased in spleen/BW ratio, only CD8+ T cells (r=−0.672, p=0.003) might be significantly decreased in spleen/BW ratio (Table 19). This correlation suggests that mouse CpG ODN is able to modulate the proportion of lymphocytes and spleen volume.

In parallel, there was correlated positively between kidney/BW ratio and BUN (r=0.703, p=0.002), Cr (r=0.571, p=0.017), or 31st week proteinuria (r=0.544, p=0.024) (Table 20). I hypothesized that enlarged kidney might be incurred by diffuse proliferative glomerularnephritis. As for the evidence of damaged kidney, there were increased BUN and Cr in serum and higher level of protein in urine.

Next on hematologic parameters, there was positively correlated between WBC and each WBC differential count: neutorphil (r=0.933, p=0.000), lymphocyte (r=0.909, p=0.000), monocyte (r=0.689, p=0.002), eosinophil (r=0.461, p=0.054) and basophil (r=0.404, p=0.096) appeared toward positive correlation, but not significant (Table 21).

In Table 22, anemia-related variables such as RBC, Hb, and Hct were negatively correlated with BUN (r=−0.800, p=0.000; r=−0.849, p=0.000; r=−0.838, p=0.000), Cr (r=−0.895, p=0.000; r=−0.928, p=0.000; r=−0.932, p=0.000), kidney/BW ratio (r=−0.431, p=0.084; r=−0.539, p=0.026; r=−0.583, p=0.014), 31st week proteinuria (r=−0.554, p=0.021; r=−0.672, p=0.003; r=−0.652, p=0.005), CD154+ T cells (r=−0.598, p=0.011; r=−0.610, p=0.009; r=−0.608, p=0.010). It refers that increased serum BUN and Cr, kidney/BW ratio, and 31st week proteinuria level with kidney damage parameters might contribute to severe anemia. Further, activated T (CD154+) cells showed negative correlation with anemia variables. It refers that high-affinity or -avidity autoantibody by activated T cell might augment immune complex binding and anti-erythrocyte autoantibody for autoimmune hemolytic anemia in SLE.

As for the correlation of between parameters of renal function test and proportion of sub-lymphocytes in spleen (Table 23), BUN, Cr, or BUN/Cr was positively correlated, whereas there was negatively correlated among spleen/BW ratio (r=−0.690, p=0.002 with BUN/Cr) and CD4+ T cells (r=−0.532, p=0.028 with BUN; r=−0.571, p=0.017 with Cr), implying that decreased renal function parameter might affect the proportion of CD4+ T cells in splenocytes and spleen volume.

Table 19. Correlation between spleen/BW ratio and each leukocyte subpopulation from spleen (n=18)

sub-lymphocytes		CD45R /B220	CD3+	CD4+	CD8+	CD4+ CD154+	CD11c+	CD80+	CD86+	I-A/I-E
spleen/BW	r	0.620	-0.466	-0.206	-0.672	0.002	0.651	0.381	0.752	0.713
	p	0.008	0.060	0.428	0.003	0.993	0.005	0.131	0.000	0.001

Table 20. Correlation between kidney/BW ratio and parameters of renal function test (n=18)

		BUN	Cr	BUN/Cr	31^{st} week proteinuria
kidney/BW	r	0.703	0.571	0.331	0.544
	p	0.002	0.017	0.195	0.024

Table 21. Correlation between WBC and WBC differential counts (n=18)

WBC diff.		NE	LY	MO	EO	BA
WBC	r	0.933	0.909	0.689	0.461	0.404
	p	0.000	0.000	0.002	0.054	0.096

Table 22. Correlation between anemia and serum renal function or activated T cells (n=18)

		BUN	Cr	BUN/Cr	Kidney /BW ratio	31st week proteinuria	CD4+ CD154+
RBC	r	-0.800	-0.895	-0.088	-0.431	-0.554	-0.598
	p	0.000	0.000	0.729	0.084	0.021	0.011
Hb	r	-0.849	-0.928	-0.222	-0.539	-0.672	-0.610
	p	0.000	0.000	0.376	0.026	0.003	0.009
Hct	r	-0.838	-0.932	-0.188	-0.583	-0.652	-0.608
	p	0.000	0.000	0.455	0.014	0.005	0.010

Table 23. Correlation between parameters of renal function test and proportion of leuckocyte subpopulations from spleen (n=18)

		Spleen /BW ratio	CD4+	CD4+ CD154+	CD8+	B220 /CD45R+
BUN	r	-0.205	-0.532	0.419	0.010	-0.051
	p	0.414	0.028	0.094	0.970	0.844
Cr	r	0.036	-0.571	0.481	-0.210	0.120
	p	0.888	0.017	0.051	0.418	0.646
BUN/Cr	r	-0.690	-0.086	0.059	0.426	-0.363
	p	0.002	0.743	0.823	0.088	0.152

V. DISCUSSION

While CpG ODN as promising immunotherapeutic has been widely used or adopted in preclinical or clinical setting (2, 3, 19), still the safety and toxicity of CpG ODN have been poorly understood. Moreover, there are scantily documented about the immunotoxicities of CpG ODN. Of potential immunotoxicities, basic immunotoxicological profiles as well as autoimmunity has been suggested as a primary priority owing to rarity of the evidenced documents. In this point, potential immunohazard induced by CpG ODN should be resolved toward wider or safer usage of CpG ODN in biomedicinal fields. Considering these background, I designed two serial, consecutive in vitro, in vivo experiment using autoimmune lupus-prone mice: optimal dosing (CpG ODN 10 μg, 4weeks) and escalated dosing (0, 10, 30, 50 μg, 8weeks).

This study indicates that optimal or escalated dosing of CpG ODN might be less immunotoxic and, rather, attenuating autoimmune indexes in lupus-prone NZB/NZW F1 female mice. Primarily, our key findings stem from *in vivo* immunotoxicological parameter proofs: no differentiality of renal histopathology, attenuating autoimmune-hematologic indexes (anemia, thrombocytopenia), and differences of nonspecific immunotoxicological profiles (spleen/BW ratio, kidney/BW ratio). Some reports have proposed and/or argued that CpG ODN might incur SLE like manifestation because of autoantibody induction against self nucleic acid (24, 107-109, 127, 132-134). However, their evidence remains variable, inconsistent and unclear depending on the modelized animal or CpG ODN dosing and therapeutic schedule. Moreover, prior to confirming autoimmune hazard triggered by CpG ODN, direct evidences about basic immunotoxicological profiles are lacking. In that context, basic immunotoxicological profiles were probed. In the first experiment, there were no

significant differences among control, non-CpG ODN and mouse CpG ODN groups: in nonspecific immunotoxicological profiles (BW, organ/BW ratio, and WBC, RBC, SGOT, and SGPT) and in evaluation profiles of glomerulonephritis (BUN, Cr, BUN/Cr ratio, renal histopathology and proteinuria). However, titer of anti-dsDNA and anti-cardiolipin Abs in mouse CpG ODN group rose three or eight-fold higher than in control group. This autoantibody induction might be elicited by potent adjuvanticity of test CpG ODN, validating the feasibility our optimal dosed model. In terms of therapeutic dose, the basis of optimal or escalated dosing and their schedule lies on our previous and cumulative data as well as mimicking or translating the schedule of current clinical CpG ODN based immunotherapy (71, 102, 105, 128). Minor benefits are that in two serial experiments, I have constructed basic immmunotoxicological profiles of TLR9 ligand by using standard mouse CpG ODN. Of note, in optimal dosing experiment, mouse CpG ODN-treated NZB/NZW F1 group displayed less severe or more attenuating index when gauging immune cell and cytokines parameter as a major index of serious SLE disease. These phenomena are partly consistent with some reports that CpG ODN revealed non-toxicity on *in vivo* experiments (135-137). Of curiosity, in first experiment, increased autoimmune antibody level failed to cause renal dysfunction or pathological lesions. In summary, first, optimal dosed-experiment to delineate the immunotoxicity of mouse CpG ODN in lupus-prone mice failed to prove the differentiality of immunotoxicity among control and CpG ODN groups in terms of clinical and immune cell function parameters. To reconfirm mouse CpG ODN-induced immunotoxicity, I initiated dose-escalating experiment, where mice were weekly i.p. treated with escalated dose of mouse CpG ODN for 8week, and observed for 12week to compare starting point of proteinuria without any intervention.

In second escalated dosed experiment, I confirmed the retarding renal disease progression triggered by escalated dose of mouse CpG ODN. For instance, cumulative reports showed that generally, about 50% of NZB/NZW F1 mice developed renal disease at eight month (124, 138, 139). Surprisingly, likewise the first experiment, there were no differentiality of immunotoxicity among control and escalating dosed-CpG ODN groups in terms of clinical and immune cell function parameters. Furthermore, while all groups showed lupus Class Ⅳ glomerulornephritis with proteinuira, control mice group manifested higher kidney/BW, and BUN/Cr, faster starting point of proteinuria as well as severe, overt anemia or thrombocytopenia than in mouse CpG ODN treated groups. Combining with two separate consistent data, these immunotoxicological profiles show that optimal or escalated dosing of CpG ODN might be less immunotoxic and, rather, attenuating autoimmune indexes in lupus-prone NZB/NZW F1 female mice. However, the underlying, autoimmune-attenuating mechanism of CpG ODN is unclear.

Based on our immunotoxicological profiles this phenomena might be explainable: First, convincing data hinted that 10 nmol (≒60 ㎍) CpG ODN treatment could stimulate granulocyte-macrophage CFUs (GM-CFUs) and early erythroid progenitors (burst-forming units-erythroid, BFU-E) toward promoting extramedullary hemopoiesis (140). Even though high dose of CpG ODN (over 100 ㎍) per mouse developed anemia in normal mice [unpublished our data] (117), this highest dosing clinically or practically is not valid.

Our first and second studies verified that CpG ODN treatment barely affected a decrease in the counts of leukocytes, erythrocytes, and thrombocytes. Contrary to our expectation, CpG ODN untreated groups manifested anemia, leukopenia/lymphopenia, and thrombocytopenia.

Further, I infer that in SLE mice model, optimal or escalated dose of CpG ODN therapy might stimulate or control extrameullary hemopoiesis.

Next, CpG ODN might induce B cell depletion or functional modulation of B and T lymphocytes in our lupus mice model. In SLE disease, the B cells were key player in developing autoimmunity and disease progress because large numbers of B cells spontaneously produce pathogenic and high-affinity autoantibody (141). Several lines of evidences showed that in autoimmune human and NZB or NZB/NZW F1 mice, increased numbers of B-1 cells are found in patients with autoimmune disease (142-146). In our first experiment, reduced B cells might be ascribed to apoptotic activity via TLR9 ligation. Emerging data provided the evidence that CpG ODN could induce apoptosis of transformed or dysfunctional B cell in vitro as well as in vivo. In this regard, splenic B cell from lupus-prone NZB/NZW F1 mice might be prone to apoptosis. Conversely, our second experiment showed that there was a decrease in the proportion of activated T (CD4+CD154+) cells in CpG ODN-treated groups compare to control group, albeit a increase in the proportion of B cells. These discrepancies about the fluctuation of B cell fraction might be attributable to differential time schedule between two experiments: in case of optimal dosing test or escalated dosing test for animal sacrifice: 8 or 20 weeks of age. In the 2nd experiment, chronic stressed B cell in lupus-prone mice might be tolerized and rarely affect or counteract CpG ODN-induced immunomodulation. Thus, I rather defined the proportion of activated T cell as a genuine autoimmune index reflecting autoimmune status. Activated T cells render B cells to produce high-affinity, avidity, specific antibodies in the autoimmune process (147-150). Given these, decreased numbers of activated T (CD4+CD154+) cells might less stimulate B cell to produce high affinity, pathogenic autoantibodies, consequently relieving SLE manifestations. Apparently, even if there were no consistent data between two separate experiments,

decreased number of B cell as well as the activated T cells might attenuate or protect development of SLE disease in lupus-prone mice.

Our experiment has another benefit to precisely guage immunotocivity of autoimmune-inducible TLR ligand such as CpG ODN, LPS, and peptidoglycan. NZB/NZW F1 mice model revealed relatively consistent, reproducible immuntoxicity of CpG ODN in SLE. Originally, New Zealand strains have an MHC-linked deficiency in the expression of the pro-inflammatory cytokine TNF-α as a human SLE without using knockout technology (125). Coupled with both experiment data, I rationally presumed that spontaneous originated NZB/NZW F1 mice might be more close to human SLE because 18 NZB/NZW F1 female mice showed significant correlation between clinical parameters of the disease as well as human SLE (Table 19-23).

VI. CONCLUSION

Collectively, these results indicate that CpG ODN might be less immunotoxic or attenuating autoimmune indexes on lupus-prone NZB/NZW F1 female mice, clinically implying that CpG ODN based immunotherapy might be justifiable in lupus-prone host. One of the limitations of this study is the small size of mice numbers, the lack of molecular data supporting immune mechanism of CpG ODN. Further studies to resolve these issues are underway.

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ABSTRACT (IN KOREAN)

Mouse CpG 올리고핵산 투여에 의한 자가면역질환 마우스(NZB/NZW F1)에서 면역독성 효과

배경 및 목적: 비메칠화된 CpG 올리고핵산은 박테리아의 핵산과 유사해 선천면역세포의 Toll-like receptor 9 를 통한 세포내 신호전달로 면역계를 조절한다. 최근 암, 알러지, 감염질환증의 난치질환 치료제로 CpG 올리고핵산의 용도가 확대되고 있으나 우려되는 독성중 면역독성평가에 대한 연구가 적어 CpG 올리고핵산의 임상적 적용에 제한점이 되고 있다. 본 연구는 CpG 올리고핵산을 치료적 용량을 투여시 자가면역질환 NZB/NZW F1 마우스에서 면역독성에 미치는 영향을 조사하였다.

연구 방법: 면역독성을 측정하기 위해서 두 단계 실험을 실시하였다. 첫번째 연구는 CpG 올리고핵산 10 μg (대조 CpG 및 마우스형/1826 CpG)을 매주 1 회씩 4 주간 투여후 일주일 뒤 마우스의 면역독성차이를 비교하였고, 두번째 연구는 마우스형 CpG 올리고핵산을 각 10, 30, 50 μg 용량을 높여 매주 1 회씩 8 주간 투여후 12 주간 관찰한 후 면역독성을 평가하였다. 면역독성을 측정하기 위해서 기본적인 체중 및 장기/체중 비율, 혈액검사 및 혈청검사를 시행하였고, 자가면역항체검사(anti-dsDNA, anti-cardiolipin), SLE 질병 진행에 따른 사구체신염을

평가하기 위한 신장조직 및 단백뇨검사, 면역 말초기관인 비장에서 백혈구의 비율차이 및 사이토카인을 측정하였다.

연구 결과: 첫번째 연구에서 기초 독성검사(체중 및 비장무게, 백혈구, 적혈구검사, SGOT, SGPT)와 사구체신염을 평가하기 위한 BUN, Cr, BUN/Cr 비율, 신장조직검사 및 단백뇨검사에서 정상범위를 유지하며 세 그룹간 차이는 없었다. 그러나 자가면역항체는 마우스형 CpG 를 투여한 군에서 증가하였으나 통계적으로 유의하지 않았으며, 항카디오리핀항체는 SLE 대조군에 비해서 8 배 이상 증가하였다. 두번째 연구에서 기초 독성검사에서 비장 (대조군 대 마우스 CpG 투여군 50 μg, $p<0.05$) 및 신장 (대조군 대 마우스 CpG 투여군 30 μg, $p<0.05$)에서 장기/무게비율의 차이가 관찰되었다. 혈액학적 검사에서 마우스형 CpG 투여군에서는 정상범위 또는 증가된 혈액학 소견을 보였으나 대조군 마우스에서는 빈혈과 혈소판 감소증이 나타났다. 대조군에 비해 마우스형 CpG 투여군에서 자가면역항체도 증가하였으나, 모든 그룹에서 SLE grade IV 사구체신염으로 판명되었으며 BUN/Cr 비율 증가 및 단백뇨가 관찰되었다.

연구 결론: 두 단계 실험에서 모두 CpG 올리고핵산에 의한 자가면역항체의 증가가 나타났으나, SLE 질병으로 이행하는 면역독성의 프로파일의 차이가 없거나 오히려 경감되는 경향을 보였다.

핵심되는 말: CpG 올리고핵산; 면역독성; 전신성홍반성낭창질환; 자가면역질환 NZB/NZW F1 마우스